The Tohoku Journal of Experimental Medicine, Vol. 56, No. 3, 1952

On the Respiratory Arrhythmias of Man as Observed Repeatedly in the Same Subjects During a Period of One Year

By

Hisao Sato

(佐藤 久夫)

(From the Laboratory of Applied Physiology, Director: Prof. K. Matsuda, and Prof. S-T. Katsura's Surgical Clinic, Faculty of Medicine, Tohoku University, Sendai)

(Received for publication, January 14, 1952)

Introduction

In a previous communication from our laboratory Matsuda and Suzuki[1] demonstrated that the respiratory arrhythmia in man is under the influences of various factors, the most outstanding of which are the cardiac rate and the depth of respiration. As to the effects of cardiac rate, tachycardia is usually accompanied by a decrease and bradycarida by an increase in respiratory arrhythmia. In other words, other factors being equal, the respiratory arrhythmia changes in the same manner as the pulse interval. The above-mentioned authors plotted the extents of the respiratory arrhythmia, measured by the difference between the pulse intervals in expiratory and inspiratory phases of respiration, against the expiratory pulse intervals, and obtained in each one of the individuals examined an almost linear relationship between two sets of values concerned. The position and slope of such a curve, however, differed from one individual to another, curves with steeper slopes being more likely found in younger individuals. For such a difference, no doubt, is to be attributed to factors other than the cardiac rate itself or respiration, the authors supposed it to be due to individual differences in the excitability of the extra-cardiac nerve centers, particularly of the vagus center.

Anyhow, it is a matter of significance to study more closely whether the mode of correlation between the respiratory arrhythmia and the pulse rate may be regarded as an individual characteristic or not. For this purpose I planned to investigate the respiratory arrhythmias in some arbitrarily selected individuals repeatedly during a considerable period of time. The results thereof I will report in this communication.

HEALTH CENTER LIBRARY

Exprerimental

Procedures

Eight adult, healthy individuals, one female and seven male, were selected for study. Their ages were from 20 to 42 yrs. They were members of our laboratory and fully accustomed to the tests by repeated previous trials.

Changes in the instantaneous pulse rate were recorded photographically by means of Matsuda's *Cardiotachograph*.[2]

Observations were carried out under resting, postabsorptive conditions of the subjects, in recumbent, sitting and standing postures successively. Any extraneous, disturbing influence was carefully avoided. For each posture a record of over three minutes' duration was taken.

The methods of computations of the extent of the respiratory arrhythmia and pulse intervals were the same as in the communication referred above.[1] The values were always expressed in seconds.

Such examinations were performed every other month in each indi vidual. The series of experiments was started in June 1949 and finished in February 1950.

Results

Although the room temperature varied considerably according to the seasons of the year, and also the respiratory arrhythmias under conditions of rest, were observed to be variable from time to time, no clear-cut tendency to indicate the presence of any seasonal variations in the respiratory arrhythmia was found (Fig. 1).

Concerning the question as to the existence of correlation between the respiratory arrhythmia and the expiratory pulse intervals, the results came out to be affirmative. All the values taken altogether from observations carried out during a period extending to nearly one year indicated an obvious interrelationship as it is shown by two examples in Fig. 2.

The curve of trend was characteristic to each individual, especially in respect to its slope to the horizontal axis. Such curves from eight individuals are represented in Fig. 3.

One of the curves, that from subject No. 8, who showed the most marked respiratory arrhythmia, ran a peculiar course in the region of longer pulse intervals. Such a phenomenon, however, seems not to be exceptional; we have some evidences that every curve takes a similar course if it be possible to be followed farther to the region of sufficiently low cardiac rates. As to this point, viz. an influence of extreme bradycardia upon the respiratory arrhythmia will be discussed in one of the forthcoming reports from our laboratory.

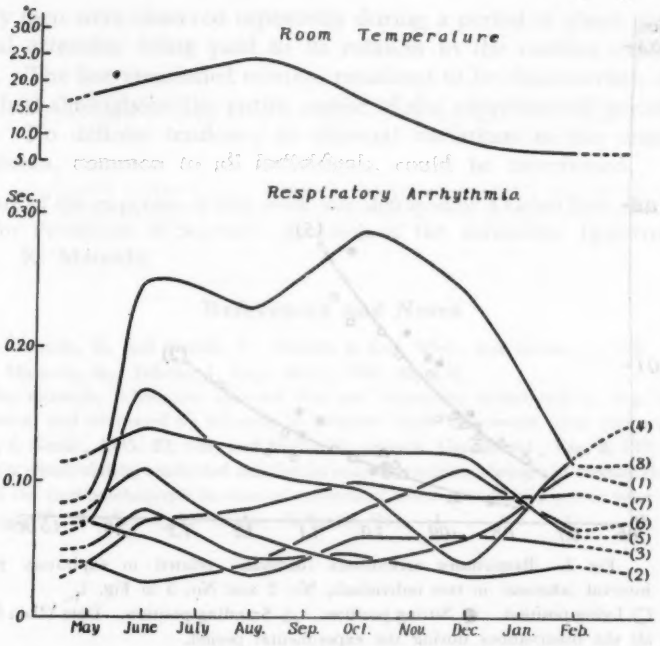


Fig. 1, Graphs showing the changes of respiratory arrhythmia during a year, (1) (2) etc, signify the individuals.

Comment

The fact that the respiratory arrhythmia stands in an relation with the cardiac rate in a manner particular to each individual, as stated by Matsuda and Suzuki, was again firmly corroborated by this experiments.

It has often been cosidered by some investigators that there is a difinite tendency to seasonal variations in the behavior of the autonomic nerves, based on various observations on animals. It is conceivable also in man. For the respiratory arrhythmia is strongly influenced by the autonomic nervous system,* it is not without ground to suppose of its seasonal fluctuations common to all individuals. But I could not obtain any evidence for such a conception.

My studies concerning the effects of a certain drugs acting on the autonomic nerves upon the cardiac rhythm of man, particularly in respect to their relation to the seasons are under progress.

Summary

1. By means of Cardiotachograph the respiratory arrhythmias of

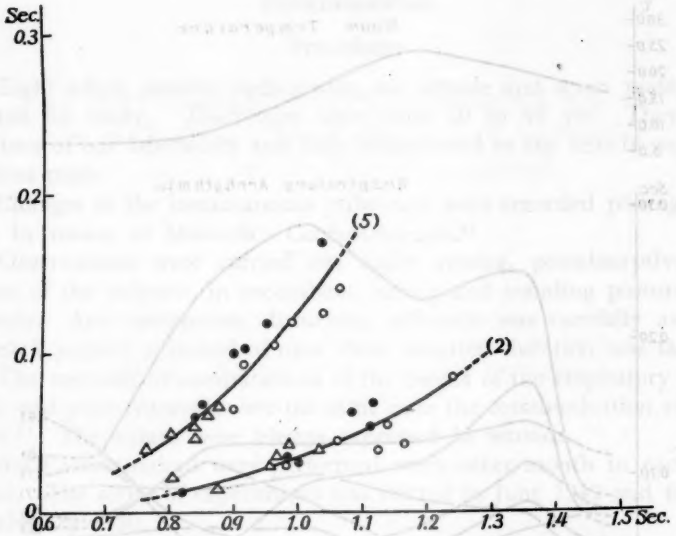


Fig. 2. Respiratory arrhythmia (ordinate) related to expiratory pulse interval (abscissa) in two individuals, No. 2 and No. 5 in Fig. 1.
○ Lying position, ● Sitting position, △ Standing position. Data taken from all the observations during the experimental period.

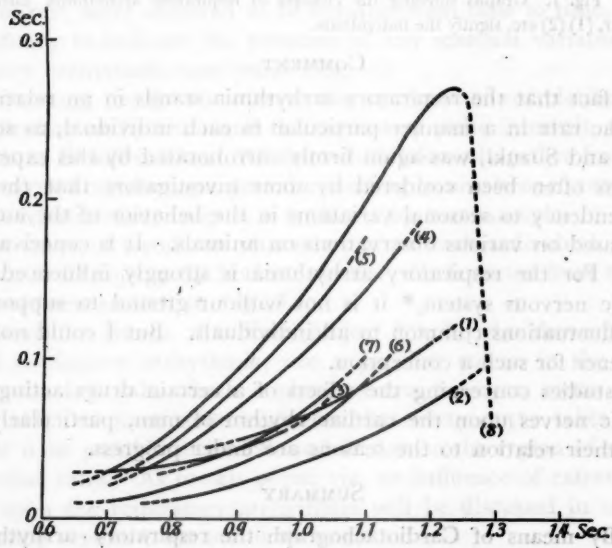


Fig. 3. Curves representing the relationship between respiratory arrhythmia and expiratory pulse interval (as in Fig. 2) of all the individuals. Numbers in parenthesis correspond to those in Fig. 1.

healthy men were observed repeatedly during a period of about one year, especial attention being paid to its relation to the cardiac rate.

2. The last mentioned relation remained to be characteristic to each individual throughout the entire course of the experimental period.

3. No definite tendency to seasonal variations in the respiratory arrhythmia, common to all individuals, could be ascertained.

Part of the expenses of this work was defrayed by a Grant from the Foundation for Promotion of Scientific Research of the Education Department of Japan. K. Matsuda.

References and Notes

1) Matsuda, K. and Suzuki, T., Tohoku J. Exp. Med., this volume, p. 199.

2) Matsuda, K., Tohoku J. Exp. Med., 1948, **49**, 246.

* For example, Schweitzer observed that the respiratory arrhythmia in dogs increased by elevation, and decreased by lowering in pressure inside the carotid sinus (Schweitzer, Z., Zeitschr. f. Kreisl., 1935, **27**, 309, and Verhandl. deutsch. Ges. Kreisl., Tag. **8**, 148). Tanemura, in a recent article, confirmed this fact in man by studying changes in cardiac rhythm by means of the cardiotachograph in cases of patients of whom the carotid nerves were removed (Tanemura, T., Tohoku Igaku Zasshi, 1949, **42**, 16).

The Tohoku Journal of Experimental Medicine, Vol. 56, No. 2, 1952

Silicosis in the Diatomaceous Earth Factories

By

Toshiaki Ebina, Yukihiro Takase and **Yoshio Inasawa**

(海老名 敏明)　(高世 幸弘)　(稲澤 嘉夫)

(From the Research Institute for Tuberculosis and Leprosy, Tohoku University, Sendai)

and **Koichi Horie**

(堀江 孝一)

(From Miyagi Labor Standard Bureau, Sendai)

(Received for publication, July 22, 1952)

It is well known that silicosis is the disease caused by inhalation of dust containing free silica for a long period and occurs often in metal-miners, workers dealing with polishing powder and glass-materials, tilers, pottery workers, coalminers, stone-masons, quarriers, metal grinders and sandblasters etc. But reports on the silicosis in workers of the diatomaceous earth factories are so rare that we could find none but that of Legge and Rosencranz.

On the one hand it is a well known fact that active tuberculous complications are found in a high percentage of silicotic cases.

Object and Method of Examination: We surveyed all of the 106 workers in diatomaceous earth factories in Miyagi-ken. The diatomaceous earth is excavated from the diatom deposits in Enda-mura, Katta-gun, Miyagi-ken.

In the factories the workers have inhaled a great deal of diatomaceous dust for a long time. The number of dusts varies from 4,600 to 3,200 in one cc. air in different factories. Dust particles are almost under 3 μ.

Results:

1) We found 12 silicosis patients (11.0 per cent) in the 106 workers in six factories dealing with diatomaceous earth at Enda by roentgenographical examinations of their chests. The 12 silicotics were graded as 8 cases in the first stage, 3 in the second stage and only one in the third stage.

2) The shortest period that incurred silicosis was 3 years and the morbidity tended to increase with the years employed. Advanced forms of silicosis above the second and the third stages are found in the group of those who had been employed 15 years or more. Morbidity here increased also with the age.

3) Except for the tendency of decrease in breathing capacity, subjective and objective symptoms were few.

4) Tuberculous complication is found only in one case of the first stage.

5) The Enda diatomaceous earth contains 76% to 86% SiO_2, which is amorphous hydrated silica in the condition of irreversible colloid and not quartz, which is generally considered as the cause of silicosis.

And so our cases are of some interest in considering the etiology in silicosis.

References

1) Legge, R. and Rosencranz, E., Amer. J. Publ. Health; 1932, **25**, 1055.

The Tohoku Journal of Experimental Medicine, Vol. 56, No. 3, 1952

Color Processes Caused by Alternating Currents in the Mammalian Retina

By

Koiti Motokawa, Kituya Iwama and **Mituru Ebe**

(本川 弘一) (岩間 吉也) (江部 充)

(From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai)

(Received for publication, January 17, 1952)

It was about a century ago that color sensations aroused by electrical stimulation of the eye were one of the most favorite topics in the physiology of vision.[1)2)3)4)] In this century, however, very little work has been done with the idea of utilizing this phenomenon for the study of color vision. This is probably because color sensations produced in this way are generally so vague that they can hardly be made use of as index for any quantitative measurement.

Motokawa and Iwama[5)] found that there appeared several minima in the strength-frequency curve obtained by stimulating the human eye with alternating currents of varying frequencies, and interpreted these minima as due to different excitabilities of retinal receptors. This interpretation was supported by a human experiment of Motokawa and Ebe[6)] who utilized the phenomenon of retinal induction for analysis of the color-effects caused by alternating currents.

In the present investigation we attempted to provide more objective evidence for the color-producing effects of alternating currents, taking spike potentials from the mammalian retina in response to electrical stimulation as the index.

Experimental

Method

As experimental materials decerebrated cats were used. A small amount of urethane (0.5–0.7 g./kg.) was injected intraperitoneally in order to avoid an increase of reflex excitability during the course of experiment. Too deep anaesthesia should be avoided, because it tends to suppress the electrical excitability of the retina.

As was described in detail elsewhere,[7)8)] the effect of colored light manifests itself in the time course of electrical excitability of the eye after cessation of the light stimulus. In the present experiment, the electrical

excitability of the eye was measured by means of a single constant current pulse lasting for 0.5 sec. which was applied to the retina through a small electrode 1 mm. in diameter inserted into the vitreous body and a large electrode placed on the injured part of the head. These electrodes were of Zn-$ZnSO_4$-agar-agar type so that polarization at the electrodes was negligible against that in tissues. The index for threshold determination was multiple spikes picked up by means of a micro-electrode of Granit's type,[9] the caliber of which was about 60 μ. This micro-electrode, together with the different stimulating electrode, was mounted on a micro-manipulator and inserted into the eye bulb until marked spontaneous discharges appeared on a screen of a cathode ray oscillograph. Responses to electrical stimulation were observed on the screen and at the same time listened to in a loud-speaker.

After a preliminary dark adaptation of about 20 minutes, the electrical threshold was determined in a similar manner to the procedure in human experiments.[7] From 10 to 20 trials were necessary for determination of each threshold value. The effect of light was expressed in terms of ζ which is defined by the formula $\zeta=100\ (E-E_\circ)/E_\circ$, where E represents the electrical excitablity after an illumination, and $E_\circ$ that at the resting level.

The accuracy of measurements depended upon the physiological conditions of experimental animals; under favorable conditions the value of $E_\circ$ remained fairly constant for a considerably long time so that the same degree of accuracy was obtained as in human experiments, but in less favorable states the value of $E_\circ$ changed so rapidly that it was found difficult to carry out such elaborate measurements as in human experiments. At the present level of our technique sufficiently favorable conditions could be realized only in about 20% of all cases.

Results

Some records of spike potentials from cat's retina are illustrated in Fig. 1. Record A represents off-discharges from a restricted number of retinal elements in response to withdrawal of white light. Record B shows spike potentials evoked by an electrical current just above the threshold, and C spontaneous discharges, that is, a control for B. We had to discriminate responses to stimulation from the background of such spontaneous activity which was observed in all cases.

Examples of ζ-time curves or excitability curves which were obtained for white light of about 200 lux as pre-illuminating light are shown in Fig. 2 (see broken curves). When yellow light was presented prior to the white light, the curve determined after removal of the white light was found to be much higher and to have a maximum at about 3 sec. just like

a curve which would be obtained by using blue light for pre-illumination (A). This phenomenon was in a previous paper[10] designated by Motokawa as "retinal color induction" which may be regarded as representing a physiological basis for successive color contrast.

It was found that an alternating current (A. C.) of 49 c.p.s. could be used as a substitute for the yellow light used in the above experiment; when the eye was first stimulated by the A. C. and then by the white light, an excitability curve of almost the same height and shape as that for the successive stimuli, yellow and white was obtained (See B). The intensity of the A. C. used was slightly above the threshold intensity of the same current for the human eye. From this experiment it follows that an A. C. of 49 c.p.s. shows an inducing action similar to that of yellow light. The waveform of A. C. seemed to play no important role in this phenomenon, for we obtained a similar result by using constant current pulses of the same frequency instead of sinusoidal A. C.

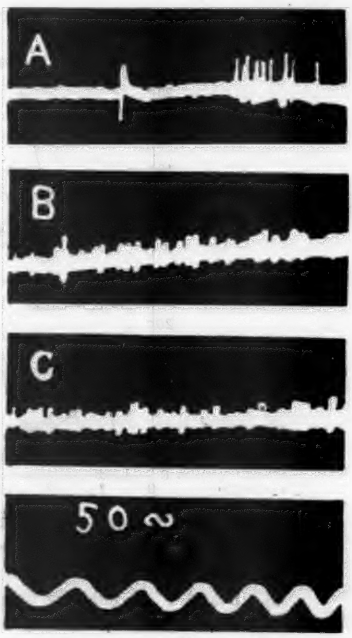


Fig. 1. Spike potentials from cat's retina. Record A: discharges in response to white light. Shock artefact preceding discharges marks the moment of turning off the light. The number of retinal elements involved is relatively small. B: discharges in response to an electric stimulus slightly above threshold. C: spontaneous discharges, control for B. The number of elements involved was greater than in A.

Another example is shown in D of Fig. 2, in which an A. C. of 31 c.p.s. was used as an inducing stimulus. The excitability curve obtained in this experiment has a maximum at about 1.5 seconds instead of at 3 sec. in B, and thus resembles the curve for successive stimuli, blue and white, which is illustrated in C. This experiment shows that an A. C. of 31 c.p.s. is equivalent to blue light. In this way we could determine functional correspondence between the frequencies of alternating currents and the wave-lengths of colored lights. The data obtained from a series of such experiments may be summarized as follows: A frequency range from 30 to 35 c.p.s. may be correlated to blue, that from 37 to 42 c.p.s. to green, that from 47 to 55 c.p.s. to yellow and a wide range over 65 c.p.s. to red. Thus, it is obvious that the color induced by A.C. varies

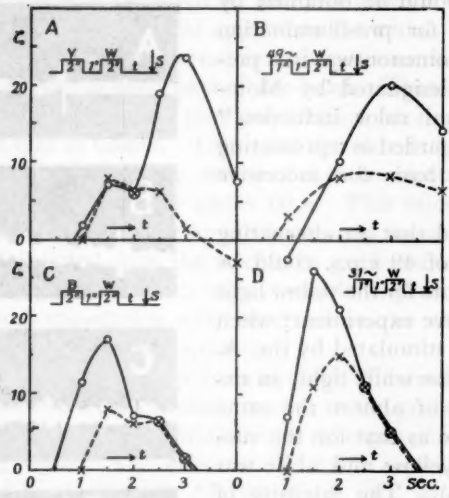


Fig. 2. Retinal induction caused by colored lights and by alternating currents. Procedure for experimentation is shown in insets where W, S and t represent white light, an electric stimulus and the interval between W and S. B and Y are inducing lights, blue and yellow respectively. A.C. used for induction are denoted by their frequencies in such a manner 49∼and 31∼. Broken curves are excitability curves for white light alone, and continuous ones are those obtained by procedure of retinal induction.

in character from blue to red in the natural order of a spectrum, as the frequency of A. C. rises.

This result was further confirmed by another series of experiments based on another principle. As was shown by Motokawa elsewhere,[11] no retinal induction takes place when another colored light complementary to the inducing one is interposed between this and the subsequent white light. If the interposed light is not complementary to the inducing one, retinal induction does not fail to appear. The after-effect caused by the inducing light is obviously neutralized by the action of the succeeding light of complementary color. In the present investigation it was found that the retinal induction caused by any colored light was neutralized by the action of an A. C. when its frequency was properly chosen. The data obtained in such neutralizing experiments are shown diagrammatically in Fig. 3. In this figure, retinal induction is expressed quantitatively by "contrast effects" and plotted against frequencies of A. C. used for neutralization. The quantity "contrast effect" is defined as the difference between the maximal ordinate of an excitability curve obtained by

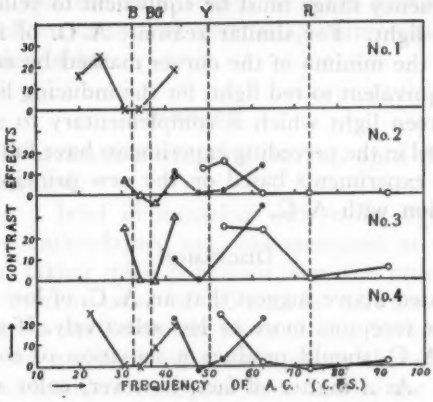


Fig. 3. Neutralizing actions of alternating currents upon retinal induction caused by colored lights. Contrast effects for yellow, red, blue and blue-green lights are expressed by crosses, triangles, dots and empty circles respectively. Broken vertical lines B, BG, Y and R show frequencies of A.C. corresponding to blue, blue-green, yellow and red lights respectively.

the procedure of retinal induction and the corresponding ordinate of the control curve for white light alone. If no retinal induction takes place as a consequence of neutralization, the value of contrast effect will be zero. On the contrary, the contrast effect will take a certain definite value characteristic of the intensity of the inducing light, if an A. C. following the inducing light has no neutralizing action upon the retinal induction caused by the preceding colored light.

In Fig. 3 the data obtained from 4 different preparations are represented at different levels in order to avoid confusion. The curves characterized by the same marks were obtained using the same inducing light. The curves marked by crosses refer to yellow inducing light and show a minimum over a frequency range from 31 to 34 c.p.s. (see No. 1 and No. 4). Since the retinal induction caused by yellow light is neutralized by blue light as well, the A.C. of this frequency range must be equivalent to blue light, and this result is in good agreement with that stated above. The curves marked by triangles (see No. 2 and No. 3) refer to neutralization of the retinal induction caused by red light. As can be seen in these diagrams, A. C. around 36 c.p.s. are effective for neutralization. Therefore A. C. of frequencies around 36 c.p.s. must be equivalent to blue-green light which is complementary to red light. The curves marked by dots represent effects of A. C. upon the retinal induction caused by blue light. Since a minimum of contrast effects is seen around 50 c.p.s.,

A. C. of this frequency range must be equivalent to yellow light complementary to blue light. For similar reasons, A. C. of frequency ranges corresponding to the minima of the curves marked by empty circles may be regarded as equivalent to red light, for the inducing light used in these cases was blue-green light which is complementary to red light. Thus, the results obtained in the preceding experiments have fully been confirmed from the present experiments based on the new principle, neutralization of retinal induction with A. C.

Discussion

The facts stated above suggest that an A. C. of low frequency stimulates retinal color receptors more or less selectively. If so, it is to be expected that an A. C. should produce a sensation of color characteristic of its frequency. As a matter of fact, however, color sensations evoked by electrical stimulation are generally so vague that it is no easy matter to identify their hues with certainty.

The question now arises as to why the color-effect of A. C. manifests itself so clearly in our experiments, despite of its vagueness in sensations. It seems to us that the vagueness is due to the extremely low saturation of colors produced electrically. The mode of action of electrical stimulation upon retinal receptors seems to be something like that of unsaturated colored light. For the phenomenon of color contrast, saturation of inducing colors seems to be of no fundamental importance, as will be seen in the following example: When a contrast figure is viewed through a sheet of thin white paper, contrast colors generally become more vivid in spite of a marked decrease in saturation on the part of the inducing colors. Similarly the degree of saturation of colors plays no important role also in the phenomenon of retinal induction, as will be demonstrated by the following experiment: Varying amounts of white light were mixed with a given quantity of red light, and the mixtures so obtained were used as inducing light to carry out experiments of retinal induction on a human subject. The values of contrast effects obtained in these experiments, to our surprise, remained almost constant, irrespective of the amount of white light added, provided that this did not exceed a certain limit. From this experiment it is obvious that the white light added contributes nothing to our retinal induction. Our contrast effects depend on the amount of monochromatic light contained in a mixture, but not on the amount of white light in it. Perception of hue becomes difficult on account of desaturation when white light is added to some degree to any colored light, but no alteration occurs in the value of contrast effect. For this reason, the phenomenon of retinal induction can be utilized for detecting the chromaticity of colored light of very low saturation without being disturbed

by its whiteness. For the same reason we have succeeded in detecting such weak color-effects caused by alternating currents as hardly perceptible as any sensation of hue.

Summary

The excitatory action of alternating currents upon the color receptors of cats was investigated.

1. Following a brief illumination with white light, the electrical excitability of the dark-adapted eye was measured at varying intervals after illumination, taking spike potentials from the retina in response to a single constant current pulse of 0.5 sec. in duration as index. The excitability showed a maximum at about 2 sec. in most cases. When the eye was exposed to yellow light and then to the white light the excitability curve was found to be higher than that for the white light alone and to have a maximum at about 3 sec. just like a curve for blue light. This is the phenomenon of retinal color induction which represents a physiological basis of successive color contrast.

2. It was found that an excitability curve of the same height and shape as that for the successive stimuli, yellow and white, could be obtained when an alternating current of 49 c.p.s. was passed through the head for 0.5 sec. as a substitute for the yellow light used in the above experiment. Thus it was proved that an A. C. of 49 c.p.s. is equivalent to yellow light.

3. In a similar manner it was shown that A. C. of 30–35 c.p.s. are equivalent to blue light, A. C. of 37–42 c.p.s. to green light, A. C. of 47-55 c.p.s. to yellow light and A. C. of frequencies higher than 65 c.p.s. to red light.

4. This result was further confirmed by another series of experiment based on the fact that the retinal induction caused by any colored light was extinguished or neutralized by the action of an A. C. of definite frequency in the same manner as by colored light complementary to the inducing one.

References

1) Müller, G.E., Ztschr. Psychol., 1897, **14**, 329.
2) Helmholtz, H., Handb. d. physiol. Optik, 3 Aufl., Hamburg and Leipzig, 1909–1911, **3**, 178.
3) Schwarz, O., Arch. Psych. Nervenkr., 1890, **21**, 588.
4) Schelske, R., Arch. Ophthalm., 1863, **9**, 39.
5) Motokawa, K. and Iwama, K., Tohoku J. Exp. Med., 1950, **53**, 201.
6) Motokawa, K. and Ebe, M., Science, 1952 in press.
7) Motokawa, K., J. Neurophysiol., 1949, **12**, 291.
8) Motokawa, K., Iwama, K. and Ebe, M., Jap. J. Physiol., 1952, **2**, 198.
9) Granit, R., Ergeb. Physiol., 1950, **46**, 31.

10) Motokawa, K., J. Neurophysiol., 1949, **12**, 475.
11) Motokawa, K., J. Neurophysiol., 1951, **14**, 339.

The Tohoku Journal of Experimental Medicine, Vol. 56, No. 3, 1952

Liver Biopsy in Children: Izumi Fever, Scarlatina and Infectious Hepatitis†

By

Yuji Sato
(佐藤 雄治)

(From the Department of Pediatrics of Prof. Ts. Arakawa and from the Department of Pathology of Prof. K. Sato, Hirosaki University, Hirosaki)

(Received for publication, January 21, 1952)

" Izumi fever "[1] is at the present time accepted as a clinical entity which is characterized by its fever curve with two or three peaks, the epidemic occurrence, and the exanthema with the characteristic localization.

The first report concerning this illness appeared in 1929 under the title of " A febrile disease with scarlatina-like exanthema which occurred epidemically among children at Kanazawa City " by Prof. Izumi. Epidemic occurrence of " Izumi fever " has been reported since then by many investigators[3][4][5][6][7][8] from various districts in Japan. According to Uchida and Kitaoka *et al.*,[4] " Izumi fever " may be differentiated from scarlet fever on the basis of the clinical findings as follows: (1) Temperature is suddenly elevated up to 38–39°C lasting for 2 or 3 days, then decreases to normal for a few days and again rises and persists over a period of 2 weeks or more; (2) exanthema with characteristic localization, it being localized chiefly on the back of the hands and around the elbows, and its development coinciding with the time of the first or the second peak of the fever; (3) absence of hemolytic streptococcus in pharyngeal secretion in the majority of cases; (4) rarity of complications and of fatality; (5) no relation between this disease and the Dick test; and (6) the negative Schultz-Charlton phenomenon.

The causative agent of " Izumi fever " appears to be still obscure, but viral theory has been advocated by Kasahara *et al.*[8]

In the present paper we shall report the liver biopsy findings of children with " Izumi fever " and compare them with the findings in cases of scarlatina or infectious hepatitis.

Experimental

Method and Material

The needle biopsy was performed with a Mitsuki needle,[9] and the

subcostal approach was employed on the mid-clavicular line in all the cases. The patients biopsied were restricted to those presenting typical clinical manifestations of " Izumi fever " or infectious hepatitis or scarlet fever respectively.

Results

Liver biopsies were carried out on 9 cases of " Izumi fever," 2 cases of infectious hepatitis and 2 cases of scarlatina.
The results of biopsies and brief clinical notes were given in Table I.

TABLE I

Biopsy Findings of the Liver in Children with Clinical Diagnosis of " Izumi fever," or Infectious Hipatitis, or of Scarlatina

No. of case	Name, sex and age (y.m.)	Date of biopsy	Lapse of days after onset of the illness	Clinical diagnosis	Biopsy findings of the liver: Changes in cytoplasm: Transparent appearance	Changes in cytoplasm: Swelling	Changes in cytoplasm: Fatty granules	Infiltration of mononuclear cells: Portal tracts	Infiltration of mononuclear cells: Lobules	Fibrosis: Portal	Fibrosis: Diffuse
1	T.S. ♂ 1.8.	10. IX. 1951	4	"Izumi fever"	++	++	−	+	+	−	−
2	T.M. ♂ 1.9.	6. XI. "	11		++	++	−	−	−	++	+
3	T.F. ♂ 5.0.	15. XI. "	11		++	++	±	−	−	−	−*
4	S.M. ♂ 7.10.	3. XII. "	11		++	++	±	+	−	−	−
5	S.N. ♀ 8.4.	14. XII. "	15		+	++	++	+	+	++	+
6	I.K. ♀ 4.6.	10. XII. "	20		++	++	±	+	−	−	−
7	J.K. ♂ 6.0.	30. XI. "	21		+	+	++	−	−	−	−
8	A.T. ♂ 4.6.	8. XII. "	33		±	±	++	+	−	+	+
9	J.Y. ♀ 3.1.	10. XII. "	30	Infectious hepatitis	±	±	++	++	++	+	−
10	K.C. ♀ 6.7.	21. VI. "	15		++	++	+	+	+	++	−
11	K.S. ♀ 6.0.	17. VII. "	7	Scarlatina	+	+	++	+	++	++	+
12	S.K. ♂ 5.0.	1. VIII. "	9		±	+	++	+	++	++	−
13	I.K. ♂ 2.7.	7. XI. "	10		±	+	+	+	++	++	−

* Edematous swelling of the connective tissues of the portal tracts and of the central veins.

Swelling of cytoplasm: Swelling of the cystoplasm of the liver cells was seen in all the cases of " Izumi fever," infectious hepatitis and scarlatina; the degree of swelling of the liver cells, however, seemed to be the most conspicuous in the cases of " Izumi fever," especially in its early stage (Cases 1–5), as compared with the other cases biopsied.

Granular or transparent appearance of cystoplasm: The cytoplasm of the liver cells was of transparent appearance in the cases of " Izumi fever," especially in its early stage (Cases 1–5). On the other hand in the cases of scarlatina the cytoplasm of the liver cells appeared to be granular, consequently more diminished in its transparency. The cytoplasm of the liver cells in the cases of infectious hepatitis also showed considerably transparent appearance, the degree of which might be evaluated to be at the middle between those of the above mentioned two diseases.

Fatty granules in cytoplasm: Fatty granules were observed in a considerable amount in the cases of infectious hepatitis (Cases 10 and 11), scarlatina (Cases 12 and 13), and in the later stage of " Izumi fever " (Cases 7–9), while they were scarcely recognized in the early stage of " Izumi fever " (Cases 1–4).

Infiltration of mononuclear cells in the lobules and the portal tracts; Infiltration of mononuclear cells in the portal tracts was seen in 5 cases out of the 9 cases of " Izumi fever " (Cases 1, 4, 5, 6, and 9), and in Cases 1 and 3 mononuclear cells were also found in the lobules as well as in the portal tracts.

In all the cases of scarlatina and of infectious hepatitis, marked infiltration of monouclear cells was seen in both the lobules and the portal tracts.

Portal fibrosis: Portal fibrosis was observed, although in a slight degree, in 3 cases of " Izumi fever " (Cases 2, 5 and 7), in which there was, also a slight evidence of diffuse fibrosis. In scarlatina and infectious hepatitis, marked portal fibrosis was observed in all the cases and was associated with a slight degree of diffuse fibrosis in one case of scarlatina (Case 11).

Edematous swelling of connective tissues of the portal tracts and of the central veins: In one case of " Izumi fever " (Case 3), edematous swelling of connective tissues of the portal tract and of the central veins was found in a considerable degree, while this finding was not observed in any of the cases of scarlatina and of infectious hepatitis examined.

Comment

Enlargement of the liver in children with " Izumi fever " was not uncommonly reported by many investigators.[4)5)6)] According to Kitaoka *et al.*,[4)] enlargement of the liver was found in 20 cases out of 22 cases examined and developed from the second or sixth day of this illness,

persisting over a period of from 2 to 10 days and disappearing coinciding with the drop of temperature to normal; and urinary output of urobilinogen was usually increased in this hepatomegalic stage.

No autopsy findings of the liver in " Izumi fever " have been reported because of the rarity of fatality from this disease, and no reports dealing with biopsy findings of the liver in " Izumi fever " have been made up to the present time.

In November 1950, we experienced about 20 cases of " Izumi fever" and, with the collaboration of K. Yamamoto Professor of bacteriology, we cultured the blood of these patients and failed to detct any evidence of known microorganisms; then the patient serum, free from any known microorganisms, was injected into the testis of guinea pigs.

Pathological findings on the guinea pigs thus infected were observed by Prof. K. Sato, of Hirosaki University, on autopsied materials successively obtained at five day intervals after the infection. Though the results were reported by Prof. Sato in 1951,* we shall present a summary of the changes in the livers: (1) Hyperemia which occurred soon after the infection and reached its maximum at about the 5th day and gradually decreased in intensity and disappeared by the 30th day of infection; (2) infiltration of mononuclear cells into both the portal tracts and the lobules began soon after infection and reached its maximum at about the 20 th day of infection and lasted until the 100th day; (3) degeneration of the liver cells, in which swelling, transparent appearance and occurrence of fatty granules in cytoplasm were included, began soon after the infection and reached its maximum at about the 5th day of infection and lasted until the 30th day.

Judging from the results of the animal experimentation above quoted, the swelling of the liver cells and the appearance of transparency observed in the early stage (4th-11th day of the illness) of " Izumi fever " (Case 1–6), might be the results of imbibition with fluid, associated with hyperemia. The edematous swelling of the connective tissues of the portal tracts and of the central veins observed in Case 3 might give important evidence supporting the consideration above mentioned.

Diffuse fibrosis, although in a slight degree, was found in 3 cases (Cases 2, 5 and 7) out of the 9 cases of " Izumi fever " and in one case of scarlatina (Case 11). This might be a consequence of prolonged maluntrition,[10][11][12][13] especially protein deficiency, commonly found in the Hirosaki area.

Summary and Conclusions

On 9 cases of " Izumi fever," 2 cases of infectious hepatitis and 2 cases of scarlatina, the liver biopsy findings were reported.

In the early stage of " Izumi fever," swelling of the liver cells with transparent appearance seemed to be the most conspicuous finding, and edematous swelling of the connective tissues of the portal tracts and of the central veins was seen in one case, while in the lafer stage, fatty granules became visible in a considerable amount in the cytoplasm.

The transparent appearance of the liver cells was also observed in the cases of infectious hepatitis but its degree seemed to be less than in those of " Izumi fever." In scarlatina, cytoplasm of the liver cells appeared to be granular, consequently it showed the least transparency among these three diseases in question.

The infiltration of mononuclear cells in the portal tracts and in the lobules was found in a considerable degree in the cases of infectious hepatitis and of scarlatina, while scarcely at all in those of " Izumi fever."

Regarding the tendency to diffuse fibrosis observed in 4 cases, it might be assumed that it probably is not directly related to the acute illness in question, but might be a consequence of prolonged maluntrition (especially protein deficiency) commonly found in the Hirosaki area.

References and Notes

1) Uchida, M., Nihon Izi Shinpo, 1951, No. 1417, 3.
2) Izumi, S., Yagazaki T., and Sugishita, N., Zika Zassi, 1929, No. 348, 50.
3) Saito, Sh., Nippon Densenbyo Gakkai Zassi, 1938, **12**, No. 6.
4) Kitaoka, M., Shishido R., and Ishii, K., Nihon Izi Shinpo, 1951, No. 1417, 8.
5) Uchida, M., Ashidate, T., Suzuki, Y. Yoshida, A., Hiraishi, H., Ugai, Sh., Iizima, T. Arima, S. and Yanuma, K. ibid., 1951, No. 1418, 9.
6) Oda, B. Fukushima, M., ibid., 1951, No. 1418, 2.
7) Kita, A., ibid., 1951, No. 1418, 26.
8) Kasahara, Sh., Ishida, K., Yoshioka, I. and Arima, A., ibid., 1951, No. 1417, 5.
9) Mitsuki, K., Tokyo Izishinshi, 1951, **66**, 1949.
10) Arakawa, Ts., Wada, Sh., and Takahashi, F.; Tohoku J. Exp. Med., 1951, **55**, 61.
11) Wada, Sh. and Arakawa, Ts., ibid., this volume, p. 47.
12) Sato, Y. and Arakawa, Ts., ibid., this volume, p. 59.
13) Masuda, K. and Aoyama, J., ibid., 1951, **55**, 1.

† Read at the General Meeting of Tohoku Medical Society, Sendai, Nov. 27, 1951.

The Tohoku Journal of Experimental Medicine, Vol. 56, No. 3, 1952

Virus Nephritis, Preliminary Note

By

Akira Sakamoto, Sakae Suzuki, Hideko Kato and
(坂本　陽)　(鈴木　榮)　(加藤英子)
Takashi Ariyoshi
(有吉　巍)

(From the Department of Pediatrics, Nagoya University, Nagoya; Director: Prof. A. Sakamoto)

(Received for publication, January 26, 1952)

We have succeeded in isolating a new virus from 5 patients of typical acute glomerulonephritis. Strains derived from different patients proved dentical. The following is a summary of our studies to date.

Experimental

Materials and Methods

In most cases fresh, bacteriologically sterile samples of patients' urine, taken on the 10th to 49th day of illness, were administered intraperitoneally to young guinea pigs (around 250 Gm.). Occasionally patients' blood served as material for inoculation. Urine, diluted with an equal amount of saline, in a dose of 2.5 cc., or 1 cc. of patient's whole blood was used. In subsequent passages the liver or kidney of the animals, which died following the injection, was consistently employed. Emulsions of the organs were strongly centrifuged and the supernatant, with or without filtration through Chamberland L_3, was administered to guinea pigs by a variety of routes (usually intraperitoneal, peroral or intranasal).

Results

Guinea pigs invariably died following a single administration mostly in about 2 weeks. Time of death varied from a day to 141 days. The animals showed no significant symptoms until several hours prior to death, when they abruptly became weak. The infection is serially transmissible to guinea pigs by means of inoculation by the organ emulsions proved free from ordinary bacteria, covering at the longest 12 months through 11 generations. When mice were tried, they did not reveal any evidence of infectious manifestations, even though large amounts of the pathogenic materials were given to them.

Routes of infection. Almost all routes of infection can be used to produce the disease and death. The following characteristic pathologic features

are seen. It is especially noteworthy that both peroral and intranasal routes proved to be the most efficient for successful infection; feeding of a 0.1% liver emulsion to animals may cause the acute infection leading to death.

Demonstration of the virus. The pathogenic virus was isolated in all 5 patients examined from the urine, and in some cases from the blood or an oral washing, the last of which was used after heating at 65 C for 30 minutes. It is of special interest that the virus can readily be isolated late in the course of the disease, contrary to the usual condition in virus disease. The infective agent increased in pathogenicity when cultured in chick embryo, especially on the chorioallantois. Occasional capillary hemorrhage is to be noted.

Symptoms and signs. Gross examination: animals tested regularly exhibited no symptoms except at the time immediately prior to death, when they showed suddenly weakness. Rarely they passed formed stools stained with fresh blood. Urine: albumin mostly postitive; red blood cells often present microscopically, white cells frequently found, but casts only occasionally. Blood picture: erythrocytes showed no significant changes, excepting that normoblasts may in a moderate number appear transitorily after the injection. White cell counts revealed slight leucocytosis or the normal range of variation, in either case with consistent preponderance of polymorphonuclear cells.

Autopsy. (a) Gross findings: hemorrhage occurs in the body cavities, occasionally causing death due to massive hemorrhage into the abdominal cavity. The most frequent site of the condition is the gall-baldder where a sanguinous fluid is seen in about one-third of the animals injected. Next comes in frequency the abdominal hemorrhage; ascites with blood-tinged exudate, occasionally with pure blood. Bacteriologic stuides of these fluids result mostly in no growth on anaerobic and aerobic cutltures. Further, gastrointestinal hemorrhage is rarely encountered. Kidneys are dark red in color with no hemorrhagic areas. No apparent edema in the skin. (b) Microscopical findings: the most significant and intensive lesions are found in the liver and kidney. Liver: there is marked hyperemia in general, and degeneration of varying degrees in hepatic cells, which predominates in the central zone with pictures of more or less severe cellular destruction associated often with hemorrhage with no evidence of cell accumulation in the interstitium or in the Glisson's capsule. Kidney: generally hyperemia is extremely marked. The glomeruli are mostly normal in size and shape and not poor in erythrocytes. In some of them, however, capillary endothelial cells are swollen and often proliferate, with occasional hemorrhage. In others the epithelial cells of the capsule may proliferate and there may be thickening and fibrinoid

degeneration of the Bowman's capsule. The principal site of renal lesions, however, is the tubule. It is particularly intensive and diffuse in the proximal convoluted portion, showing marked swelling of the endothelial cells, and, in subacute cases, advanced fatty, granular or albuminoid degeneration. Casts may be found. There is no evidence of cell infiltration. In brief, essentaial features of this infection manifest themselves as the picture of nephrosis, associated with localized glomerular changes already in an early stage of the disease. The spleen reveals some findings of splenitis with some reticuloendothelial responce, while marked hypertrophia and hyperplasia of the reticuloendothelial cells may often be encountered in the lymph nodes.

Acute symptoms and death of animals infected are to be attributable to liver damage, nephrosis and exudative hemorrhagic effusion in the body cavities. The above mentioned gross and microscopical changes in acute cases are considered suggestive evidences for the presence of a specific virus, causing widespread capillary damage with systemic involvement of the whole body.

Immunologic studies concerning the virus and on the infected patients are in progress.

Summary

A new virus was demonstrated by means of the urine or blood taken from patients with typical acute glomerulonephritis. The infection is transmissible to guinea pigs in series with fatal consequence. Some of the properties of the agent and the essential pathologic findings of animals infected are presented.

The Tohoku Journal of Experimental Medicine, Vol. 56, No. 3, 1952

On the Histology of Ductulus Lacrimalis in Adult, Especially on Its Innervation

By

Koichi Tsuda
(津田 光一)

(From the Anatomical Laboratory of Prof. H. Seto, Faculty of Medicine, Tohoku University, Sendai)

(Received for publication, January 31, 1952)

With regard to the histological study of the ductulus lacrimalis in man especially on its innervation it would be next to impossible to find literatures both here and abroad, only with the exception of a report by Yokomatsu[12] (1931) written very simply and occupied by somewhat doubtful things. Recently Seto[9] made public in detail the results of his study on the innervation of the eye-lid in adult, but without making any revference to the innervation of ductulus lacrimalis.

Therefore, in order to solute this problem I made the materials taken from 6 adult cadavers in 40μ vertical and transversal frosen sections after long fixation in 10% neutral formol and stained with the Seto's silver impregnation. The results obtained by the detailed examination through a great many of quite beautifully stained preparations are as follows.

Experimental

Fine Structure of Ductulus Lacrimalis

Before going into the detailed description of the innervation of the ductulus lacrimalis in man I would like to spend a few words about the histological pictures of its mucous membrane. Because generally the distribution or development of sensory nerve fibres in the mucous membrane is closely related to the states of its histological structures, as many works from our laboratory clearly indicate, but the histology of the ductulus lacrimalis has been only studied by a few investigators,[4][5][8][10][11] so that there are remained still many things not clarified yet.

The epithelium of the so-called vertical duct of the ductulus which takes origin from the punctum lacrimale (Fig. 1) is represented by a continuation of the non-cornificated stratified flattened epithelium of the margo palpebralis (Seto).[9] To my surprising, however, this epithelium increases suddenly in thickness to become about twice (ca. 80μ) as thick as that of the lid-edge, though it varies more or less according to the different

places. The epithelium of the margo palpebralis contains the granules of melanin pigment, but the epithelium of the ductulus has none of them. Besides, according to Eisler,[2] Schirmer[3] and Lauber[4] the uppermost layer of the epithelium shows a desquamation, but in my view consists of nucleated flat cells and is not desquamative, the surface being smooth.

Fig. 1. Obersight on the innervation of the vertical duct of ductulus lacrimalis in adult. Sagittal section. s plexus submucosus; w, w' special contorted terminatios found in the subepithelial connective tissue of the inner lid-edge and the vertical duct; m motor nerve fibres for m. obriculairs oculi; p puncutm lacrimale; c lamina propria of tunica conjunctiva palpebrae. Seto's silver method, X 50, reduced to $\frac{1}{2}$.

The surroundings of the epithelium are represented as a fibrous connective tissue layer being rich in elastic fibres and arranged relative densely and mainly circularly. This layer is in general narrower than the epithelium and divided into lamin apropria and tunica submucosa, though the boundary between both of them is not cleared because of lacking of lamina muscularis mucosae. It would be here most emphasized that the connective tissue of the lamina propria shows scarcely formation of papillae for the epithelium, so that the basement of the epithelium is nearly showed as a smooth surface. This actually means, as many works of our laboratory clearly show, that the mucous membrane showing no formation of papillae largely is not favoured with sensory nerve elements. In fact as described later, the mucous membrane of this area is for instance in comparison with that of the margo palpebralis and etc. far destitute

of sensory fibres.

Besides, in this lamina propria in places is recognized the infiltration of lymphocytes, though a few. According to the reports of the authorities enumerated above also in the stratified flattened epithelium of the ductulus goblet cells are found in places, but in my view the existence of such cells has never been recognized, as also in the same epithelium of mucous nature in other various parts of the body such a phenomenon should never take place.

Next, concerning the horizontal duct of the ductulus it is very interesting that it shows various changes of the histological pictures more and more as it approaches the saccus lacrimalis. Namely the beginning of the horizontal duct is histologically more or less similar to the vertical duct, but soon after in the lamina propria appears the aggregation of lymphocytes the growth of which increases with the approach of the lacrimal sac. It becomes finally to a lymph nod with a germinal center or occasionally to a aggregated lymph nod which envelops from the surroundings the epithelium of the duct, as does in processus vermiformis. The latter in general is formed near the opening into the lacrimal sac. These special areas naturally consist of reticular connective tissue, being surrounded by the fibrous membrane composed of collagenous fibres running circularly.

The changing of the epithelium of the horizontal duct attracted my attention particularly. It is based upon the changing of the stratified flattened epithelium into the stratified columnal epithelium in a scattered manner, increasing more and more as the duct approaches the lacrimal sac. This fact seems, as I know, not to have been reported by any one. On this problem it is above all interesting that the changing of the epithelium largely goes parallel to the formation of the lymphatic tissue described above. But, as even the epithelium of the mucous membrane having occasionally lymph nods is represented by the stratified flattened epithelium, the formation of the lymphatic tissue is considered to take place prior to the changing of the epithelium.

This changing phenomenon of the epithelium depends upon that the flat cells of the uppermost layer of the stratified flattened epithelium change into the columnal cells. Therefore, there is no difference in thickness between both epithelia, but near the lacrimal sac we often see the appearance of the far thinner columnal epithelium represented by only 2 or 3 layers of epithelial cells. In this thinn columnal epithelium very often we can find a large number of lymphocytes penetrating from the surrounding lymphatic tissue. It is probably considered that they will finally wander into the lumen of the duct in order to show phagocytosis as do those pentetrating into the epithelium of the crypts of various kinds of tonsils.

The horizontal duct often shows an extremely characteristic form near the opening into the lacrimal sac. This is quite different from the so-called valve-formation recently found by Ashikaga[1] in the mucous membrane of the same opening and somewhat similar to the thickning of the initima and media found in the small arteries in the thyroid, the external genitals and etc. As shown in Figs. 2 and 3 the bud- or fungi-formed thickning of the mucous membrane strongly projecting into the lumen of the duct is in general filled with the well-developed lymphatic tissue, the epithelium consisting of the thin stratified cloumnal epithelium already mentioned. Physiologically interpreted, this specific formation may be thought to exist to let the lymphocytes display their duty in full in order to catch the harmful objects especially bacteria in the tear flowing in the lumen of the duct.

Fig. 2

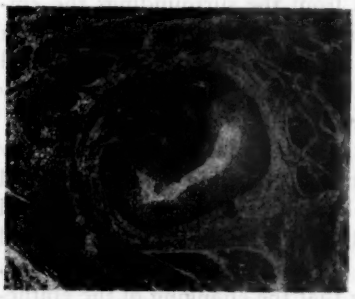


Fig. 3

Fig. 2. Bud-formed thickning of the mucous membrane projecting into the lumen found near the opening of the transversal duct into the lacrimal sac. l lymphatic tissue; c thin stratified columnal epithelium; f thick stratified flattened epithelium; b subepithelial connective layer; m m. obriculairs oculi. Same staining, Photo ×40.

Fig. 3. Fungi-formed thickning of the mucous membrane found in the same place as in Fig. 2. Marks are also the same as in Fig. 2. Same staining, Photo ×40, reduced to ½.

In the neighbourhood of the opening of the duct into the lacrimal sac is developed the lymphatic tissue with germinal centers remarkably by which the surroundings of the duct are often enveloped completely like in the lacrimal sac. The development of the fibrous connective tissue in the tela submucosa becomes also stronger.

As the ductulus lacrimalis penetrates in the eye-lid m. orbicularis oculi, the muscle fibres run to the vertical duct perpendicularly and to the horizontal duct parallel. But, in the beginning of the vertical duct they surround the duct more or less circularly, showing the appearance of a sphincter, somewhat imperfect as it.

Innervation of Ductulus Lacrimalis

Concerning this problem we have only one report by Yokomatsu[12] obtained from an infant of 3 days after birth by Cajal's method. But this work is so simply described that there are nerve elements showing a very poor development in the connective tissue of the ductulus wall, a very few of their branches terminating as intraepithelial fibres. And of course he has not given on the nature of the nerve fibres any interpretation.

Now, the nerves especially the sensory fibres for the ductulus are originated in n. infratrochlearis from n. nasocilialis of n. trigeminus, e.i. it branches into rr. palpebralis superior et inferior and distribute with their many subbranches in the inner part of the eye-lid containing ductulus. These nerves are chiefly composed of medullated thick sensory fibres, but containing a few of non-medullated minute vegetative fibres. The latter may be considered probably to take origin from the anastomosing branches of the vegetative especially sympathetic nerves to the n. trigeminus. In addition to this the wall of the samll blood vessels in subepithelial connective tissue is accompanied by the vegetative nerve plexus.

Since beneath the epithelium of ductulus is scarcely seen formation of papillae, its mucous membrane was at once supposed to be not so favoured with sensory fibres, as already noticed. Truely with high power in detail examined, the development of the sensory elements is not so remarkable by any means, but can not be said also to be so much poor, e.i. they are here developed in a medium degree. By the way, in comparison of the vertical with the transversal duct the sensory fibres are seen more richly in the latter than in the former generally in proportion to the development of the subepithelial connective tissue. As the connective tissue of the transversal duct increases gradually with the approach of the lacrimal sac, the sensory fibres also show a better development near the lacrimal sac.

The relatively fine nerve bundles running through the muscle layer pass in the tela submucosa into the nerve plexus distributing chiefly circularly (Fig. 1). This formation is in general very weak, but according to the places especially in the transversal duct often represented as a relatively strong plexus. The sensory nerve fibres originated in this plexus advance mostly forward into lamina propria and without forming any remarkable plexus here pass into the terminations of various forms as mentioned next.

Now, in the ductulus are with my detailed examination neither intraepithelial nerve fibres nor corpusclar terminations, though Yokomatsu[12] cited as a kind of terminations a few of intraepithelial fibres penetrating its epithelium. Since the epithelium of the ductulus consists

of non-cornificated stratified epithelium, it is naturally considerable that sensory fibres as a rule can enter this epithelium, as a number of works of our laboratory sows evidently. Therefore, I have examined in detail as I could, but the sensory fibres in the ductulus wall find all their terminations only in the lamina propria as described later, without advancing into the epithelium by any means. Namely, even the non-cornificated stratified epithelium do not always allow the entering of sensory fibres, as does not the epithelium of oesophagus (Sada).[6] Then it is doubtlessly considered that the so-called intraepithelial fibres by Yokomatsu[12] are nothing more than artifacts caused by his imperfect silver staining.

Also from the physiological viewpoint the presence of corpusclar terminations must be denied in the ductulus. In fact, with my detailed examination there are never seen in any part of its connective tissue layer such corpusclar terminations as the Meissner's tactile bodies and genital nerve corpuscles, to say nothing of the Pacinian bodies.

The sensory nerve fibres seen in the ductulus wall are mostly composed of thick and medium thick medullated fibres, but exceptionally partly of a few thin medullated fibres. The terminal mode of these sensory fibres is divided into about two types e.i. the simply running or non-contorted and the specific or contorted terminations.

Non-contorted terminations:—The sensory fibres are mostly represented by medium thick fibres, as shown in Figs. 4 and 5, but often by considerably strong fibres (Figs. 6 and 7). These terminal fibres have generally the relatively equal thickness, showing the variety of thickness only very rarely. By the way, these terminations are not special terminations existing only in the ductulus wall, because the terminations of this sort are found widely in various parts of the body. But, the characteristic of these terminations in the ductulus consists in that the terminal fibres end nearly always beneath the epithelium or often in close contact with the basis of the epithelium.

These terminations are divided roughly into the unbranched and the branched terminations, and the latter furthermore into the simple and the complicated forms. The unbranched terminations are most simple endings in which a medullated fibre runs a relatively straight course after losing its medullary sheath, finally to terminate beneath the epithelium dully or sharply, as Fig. 4 shows. Besides, in this figure is seen the presence of the end-apparatus of vegetative nerve fibres e.i. vegetative terminalreticulum (Stöhr) going with the sensory fibre and generally distributing extensively in the connective tissue layer.

Special contorted terminations:—They exist, unlike the non-contorted terminations only in the ductulus lacrimalis and not in other places of the body, as comprehended from a number of works of our laboratory.

Fig. 4

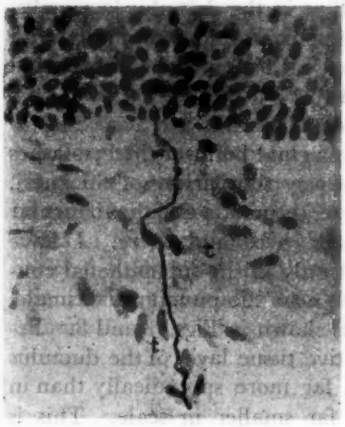


Fig. 5

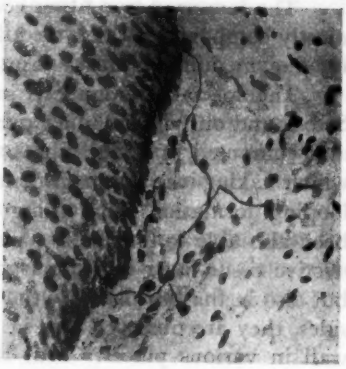


Fig. 6

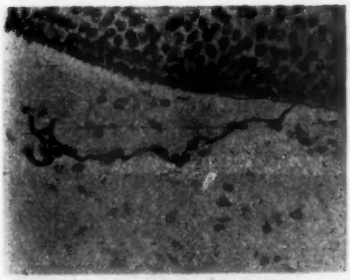


Fig. 7

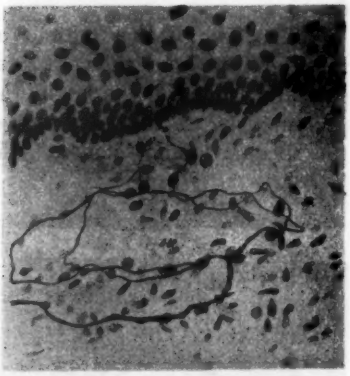


Fig. 4. Unbranched sensory termination found near the beginning of the vertical duct of ductulus lacrimalis in adult. e epithelium; t vegetativ terminalreticulum. Same staining, X 500, reduced to 2/3.

Fig. 5. Simple branched termination found in lamina propria of the transversal duct of ductulus lacrimalis in adult. Same staining, ×500, reduced to ½.

Fig. 6. Ditto. Same staining, ×500, reduced to 2/3.

Fig. 7. Somewhat complicated branched termination found near the beginning of the vertical duct of ductulus lacrimalis in adult. Same staining, ×500, reduced to ½.

Therefore, I would call them special terminations. But, the margo palperbralis is exceptive, because it makes not only the origin of the ductulus, showing the histological similarity to the latter, but also it is in fact the local where the most typical ones of these terminations exist (Se-

to).[9] Namely, these contorted terminations are very specific terminations found only in the margo palpebralis as well as in the ductulus lacrimalis.

The sensory nerve fibres concerning these contorted terminations are mostly composed of thick medullated fibres which often are accompanied by fine medullated fibres (Figs. 11 and 12).

The special terminations in the margo palpebralis are according to Seto found only in the inner part from the original point of the eye-lashes and composed of strong fibres showing scarcely the variety of thickness, which taking the looping contorted course terminate in the irregular looping formations containing special nuclei here and there. I have been also able to find their typical ones not only in the subepithelial connective tissue layer of the margo palpebralis near the punctum lacrimale, but also in the beginning of the ductulus, as shown in Figs. 1 and 8. Besides, they are observed also in the connective tissue layer of the ductulus wall in various places. Mut they spread far more sporadically than in the margo palpebralis and are generally far smaller in scale. This is above all more impressionable in the vertical duct than in the transversal duct.

The special terminations are in the ductulus wall further divided into the simple and the complicated ones. For instance, Fig. 8 is a simple special termination found in the beginning of the vertical duct and represented by thick fibres relatively rich in variety of thickness and taking the specific looping course. Fig. 9 shows a complicated special termi-

Fig. 8

Fig. 9

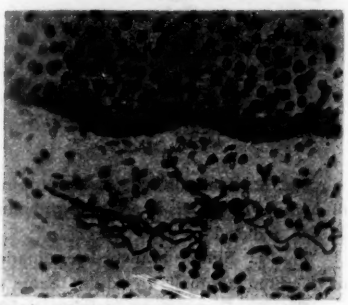


Fig. 8. A simple (left) and a complicatd form (right) of special contorted terminations found in the beginning of the vertical duct of ductulus lacrimalis in adult. Same staining, ×360, reduced to $\frac{1}{4}$.

Fig. 9. Relatively complicated special contorted termination found near the opening of he transversal duct of ductulus lacrimalis in adult. Same staining, ×500, reduced to 2/5.

nation found near the opening of the transversal duct whose fibres are arranged in relatively complicated manner and spreading in the wide

territory. This formation bears a striking resemblance to the looping termination in the margo palpebralis described by Seto[9] (his Fig. 6) and the nature of the terminal fibres is also of thick fibres having the smooth surface and showing not so remarkable vriety of thickness.

Some of the specific contorted terminations often terminate glomerularly. But they are never so complicated ones as seen in the margo palpebralis (Seto),[9] but always very simply formed. For instance, Fig. 10 shows a very simple glomerular formation belonging to the special termination found near the opening of the transversal duct. The stem fibres of this termination can not be done clearly, it might be, however, easily thought from the thick fibres in the terminal formation to take the origin from a specially thick sensory fibre. Thus, the special glomerular terminations are recognized also in the ductulus lacrimalis, but never so richly.

Fig. 10

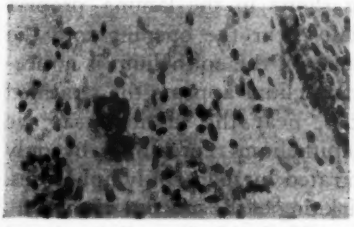


Fig. 11

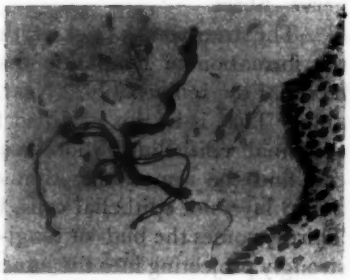


Fig. 12

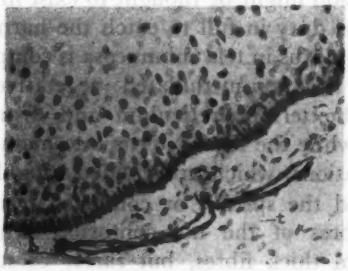


Fig. 10. Simple glomerular termination belonging to a kind of special contorted terminations found in lamina propria of the transversal duct of ductulus lacrimalis in adult. Same staining, ×600, reduced to 2/5.

Fig. 11. A variety of special contorted terminations composed of strong fibres accompanied by thin fibres found in lmaina propria of the transversal duct of ductulus lacrimalis in adult. Same staining, ×500, reduced to 2/5.

Fig. 12. Ditto. t vegetative terminalreticulum. Same staining, ×500, reduced to 2/5.

Besides, as a kind of the special terminations exist the unformed terminations which are composed of very thick fibres often rich in variety of thickness and showing not so remarkable winding course. For instance, as shown in Figs. 11 and 12, it is the very unformed terminations which are formed direct beneath the epithelium and consist of thick fibres showing the variety of thickness in places. Furthermore they are often accompanied by some of thin sensory fibres which terminate also with a unformed terminal mode.

Summary

The vertical duct of the ductulus lacrimalis has a non-cornificated flattened stratified epithelium like the margo palpebralis, which is, however, about twice as thick as the epithelium of the latter. The subepithelial fibrous tissue shows for the epithelium scarcely formation of papillae. This fact indicates that this mucous membrane is not so favoured with sensory nerve fibres.

The transversal duct of the ductulus shows in the lamina propria the formation of lymphatic tissue, especially near the opening into the lacrimal sac remarkably. Next, the changing of the epithelium is noticeable. That is in the stratified flattened epithelium appears the stratified columnal epithelium sporadically. Besides, near the lacrimal sac we can find the remarkably thin columnal epithelium represented by only 2 or 3 layers of epithelial cells, being penetrated by many of lymphocytes. Thirdly comes the bud- or fungi-formed thickning of the mucous membrane strongly projecting into the lumen of the duct, filled with the well-developed lymphatic tissue and having the epithelium of thin stratified columnal nature. This formation might be thought to exist in order to let the lymphocytes display their duty in full to catch the harmful objects.

The weak nerve plexus in tela submucosa is composed of many medullary sensory and a few non-medullated vegetative fibres. The latter finally become to the terminalreticulum and distribute widely in the subepithelial tissue, while the former terminate mainly in lamina propria with various terminations, which are divided roughly into the unspecific or non-contorted and the specific or contorted terminations.

The sensory fibres of the non-contorted terminations are mostly composed of medium thick fibres, but rarely of strong fibres, show in general the equality of thickness and terminate sharply or dully direct beneath the epithelium or in direct contact with the basis of the epithelium. The terminations are according to their complexity subdivided into the unbranched and the branched terminations, furthermore the latter into the simple and the complicated forms.

The contorted terminations are seen only in ductulus lacrimalis and

margo palpebralis (Seto).[9] Their sensory fibres are mostly strong medullated fibres, being often accompanied by small medullated fibres, represent scarcely the variety of thickness and pass into irregular looping terminal formations containing special nuclei here and there. The terminations are further divided into the simple and the complicated forms according to their scale. The former are mostly found in the vertical duct, the latter chiefly in the transversal duct.

Some of the specific terminations are represented as the simple glomerular formations, though a few. Besides, as a kind of the specific terminations sometimes exist the unformed terminations composed of strong fibres rich in variety of thickness and showing not so evident winding course, too.

References

1) Ashikaga, Nippon Ganka-Gakkai Zassi, 1940, **29**, 67.
2) Eisler, Kurzes Handbuch der Ophthalmologie, I, J. Springer Berlin, 1930.
3) Gray, Anatomy, Longmans, Green and Co. London, 1923.
4) Lauber, Möllendorff's Handbuch der mikr. Anat. des Menschen, III/2, J. Springer Berlin, 1928.
5) Rauber-Kopsch, Lehrbuch der Anatomie des Menschen, III, G. Thieme Leipzig. 1940.
6) Sada, Psychiatria et Neurologia Japonica, 1942, **34**, 164.
7) Sakai, Tohoku Igaku Zassi, 1944, **34**, 164.
8) Shirmer, Handbuch der Augenheilkunde, I/2, J. Springer Berlin, 1941.
9) Seto, Tohoku Igaku Zassi, 1946, **39**, 1.
10) Stöhr, Lehrbuch der Histologie, G. Fischer Jena, 1940.
11) Szymonowicz, Lehrbuch der Histologie, G. Thieme Leipzig, 1924.
12) Yokomatsu, Kaibogaku Zassi, 1931, **4**, 1321.

The Tohoku Journal of Experimental Medicine, Vol. 56, No. 3, 1952

Biochemical Studies on Carbohydrates

CLVII. On the Carbohydrate in Casein

By

Hajime Masamune and **Masahiro Maki**

(正宗 一) (眞木 正博)

(From the Medico-chemical Institute, Tohoku University, Sendai)

(Received for publication, February 3, 1952)

As to the carbohydrate in casein, Sörensen[1] announced once from the time-curve of an orcinol reaction that it is galactose, although she later on retracted[2] the claim because of the inaccurateness of the colorimetry due to the fluorescence by tryptophane. More recently Haneda and others[3] pointed to hexosamine (0.30%) and mannose (0.39%) as sugar components of this protein. These authors estimated hexosamine by Hamasato-Akakura method on one hand, and, on the other, they made a rough identification of the non-amino sugar from the analytical values obtained by the indole-, orcinol- and diphenylamine (I)- method.

Both the groups of investigators subjected casein immediately to analysis, which does hardly allow to warrant a definite conclusion in such a protein with a very small carbohydrate content. First in 1948, Inoue and Kudo[4] disintegrated the protein by the aid of pancreatin as well as baryta and believed to have isolated N-D-glucosido-D-arginine and N-D-glucosido-D-arginyl-L-tryptophanyl-L-tyrosine. However, what they put forward appears to us quite questionable for the reasons: 1) we have never before met with glucose nor with any hexosamine-free carbohydrate conjugating with an amino acids-grouping to build up a protein. So to check their results, we followed their baryta procedure of preparation and further purified the product in two somewhat different ways described in the experimental part.

The two specimens obtained were resembling regarding composition (hexosamine 12.9%, 12.2%; non-amino sugars as an equimolecular mixture of mannose and galactose 59.5%, 55.6%; acetyl 0.0%, 0.0%; ash 0.0%, 0.1%; iodine use as glucose 4.3%, 4.0%) and optical rotation ($[\alpha]_D = -56.5°, -54.8°$ (in 0.01 N NaOH)), and by paper partition chromatography of their acid hydrolysates they were revealed to contain glucosamine, galactose and mannose as sugar components, and aspartic and glutamic acid, glycine, alanine, proline or hydroxyproline, methionine or valine, and leucine or isoleucine as amino acid components. Neither

glucose nor arginine nor tyrosine was detected. The tube-tests showed the absence of tryptophane, too. Which of those amino acids in the specimens is attached immediately to the carbohydrate residue is not predicted at present, but it is of meaning that those amino acids together with mere serine and threonine in addition constituted the amino acids grouping in an acetyl derivative of the Group **A** carbohydrate from pig stomach mucus, as will be published elsewhere.

One of the specimens above was applied moreover to electrophoresis which proved it practically homogeneous.

Experimental

Separation of the Carbohydrate from Casein Molecule

140 g. of cow milk-casein prepared according to Hammarsten were fine pulverized and boiled for 6 hours with 1.4 *l.* of 10% $Ba(OH)_2$ under return condenser, avoiding the contact with CO_2. When cold, the grayish sediments were filtered off, and the brown filtrate was bubbled with CO_2-gas and again filtered, followed by washing the bulk on the funnel with hot water (200 cc.). The filtrate and washing were freed quantitatively from barium with just sufficient H_2SO_4, distilled in vacuo to about 0.7 *l.* and after alkalizing to a pH of 9.4 with 150 cc. of 10% ammonia and adding 320 cc. of the saturated lead subacetate (Additional lead subacetate caused only insignificant precipitation), stood for 1 hour. The precipitate was collected on a Büchner funnel and stirred up in 300 cc. of water, then the suspension was acidified to pH 4.2 with acetic acid (20%) and filtered. To the filtrate was added again 10% ammonia to pH 9.4. The yellowish precipitate separated was washed with a dilute ammonia of the same pH. Subsequently it was agitated in 400 cc. of water, warmed to 60°C and bubbled with CO_2. The precipitate was washed with 100 cc. of hot water on a suction funnel and the filtrate and washing were thrown down with 37 cc. of a 10% mercuric sulfate solution in 5% H_2SO_4. Stood for 6 hours and filtered. And the filtrate was liberated from Hg with H_2S and from H_2SO_4 with the saturated $Ba(OH)_2$ and inspissated by vacuum distillation to a syrup. This was dehydrated with an alcohol-ether (1:2 by volume) mixture, washed with ether and dried to constant in a desiccator. 1.5 g. of a hygroscopic powder with yellowish shade were yielded.

In a second run, started with 860 g. of the material, were given 8.5 g. of a similar substance.

The products were put together—**Cr. Ch.** Biuret, Molisch, Elson & Morgan and Fehling positive. Osaki & Turumi reactions, direct and indirect, negative. By the direct, orange coloration occurred but no

absorption band was seen within the λ range from 450 to 700 mμ. Tyrosine (a modified Millon) was detected but not tryptophane (Rhode; Hopkins & Cole) nor arginine (Sakaguchi). N 11.5%. Hexosamine 0.77%. Non-amino sugars: a) as expressed as galactose, indole method 3.8%, orcinol method 3.9%, diphenylamine method I 6.6%; b) as expressed as mannose, indole method 4.1%, orcinol method 4.1%, diphenylamine method I 3.5%. Ash 8.8%.

Purification

1) 3.0 g. of Cr. Ch. were taken up in 150 cc. of water. After addition of 1.5 g. trypsin (Kahlbaum) and bringing the pH to 9.0 with Na_2CO_3 (1 *N*), the mixture was placed in a thermostat at 37°C for 4 weeks, adjusting the pH from time to time to the original. A trace of an insoluble matter was centrifuged off and the supernatant neutralized (with 0.1 *N* HCl), heated at 100°C for 15 minutes and distilled in vacuo to 7 cc. The thick solution was acidulated (to pH 6.0) and vigorously exhausted with butanol in a Dakin apparatus for 36 hours. And then the solution was evaporated in vacuo to dryness and kneaded with 20 cc. of glacial acetic acid (98%) added in portions. The centrifugate was further washed with three 10 cc. portions of the glacial acetic acid and then with ether thoroughly and dried. 202 mg. of a yellowish, biuret-negative powder were given here.

It was electrodialyzed, as dissolved in 10 cc. of water (A trace of an insoluble part discarded), in a Pauli apparatus (200 volts) against changes of water for 3 days. Some precipitate was rejected and the mother fluid distilled in vacuo to a syrup, treated with abs. alcohol and ether and dried —a yellowish powder (**Ch. I**). 140 mg. was the crop.

2) Another 5.3 g. portion of the crude carbohydrate was subjected to a similar procedure of purification without initial digestion with trypsin. The amount of the product (**Ch. II**, a white powder) was 203 mg.

Properties of the Final Products

The purified substances resembled each other in qualitative-chemical aspect. Molisch strongly positive. Fehling and Elson & Morgan test resulted also in positive. The sequels of the Osaki & Turumi tests were similar as in the crude product. Ninhydrin positive, though faint. Biuret negative. Tyrosine, tryptophane and arginine were not proved present.

Solubilities. The two specimens showed a marked difference. Within some weeks after preparation, Ch. I dissolved in plain water and 0.01 *N* NaOH transparently, but Ch. II formed only an opaque and a translucent solution in water and in 0.01 *N* NaOH respectively. After preservation for half a year in a desiccator, the latter specimen was found partly in-

soluble even in the alkali, while the former still dissolved readily.

Analysis. See Table I.

TABLE I

Composition of Preparations of the Carbohydrate in Casein

Analysis	In per cent		In equiv. per equiv. wt.	
	Ch. I	Ch. II	Ch. I	Ch. II
N	2.0	2.1	2.0	2.2
Hexosamine (glucosamine)	12.9	12.2	1.0	1.0
Non-amino sugars				
As galactose:				
indole m.	57.6	54.0		
orcinol m.	54.8	54.4		
diphenylamine m. I	81.7	72.5		
As mannose				
indole m.	62.0	59.4		
orcinol m.	60.0	58.1		
diphenylamine m. I	40.8	35.0		
As an equimolecular mixture of galactose and mannose				
indole m.	59.8	56.7		
orcinol m.	57.4	56.3		
diphenylamine m. I	61.3	53.8		
Mean	59.5	55.6	4.6	4.6
Acetyl	0.0	0.0		
Iodine use as glucose (Mol. wt. calc. fr. the iodine use)	4.3 (4130)	4.0 (4500)		
Ash	0.0	0.1		

As will be recorded below, galactose and mannose are involved in the carbohydrate, and the intensities of the colors developed by the three different reagents nearly coincided as expressed as an equimolecular mixture of those hexoses.

Optical rotation in 0.01 N NaOH

$$\text{Ch. I: } [\alpha]_D^5 = \frac{-0.35 \times 100}{0.62 \times 1} = -56.5°.$$

$$\text{Ch. II: } [\alpha]_D^7 = \frac{-0.23 \times 100}{0.42 \times 1} = -54.8°.$$

Paper partition chromatography. 1) Sugar components. 60 mg. each of Chs. I and II were hydrolyzed with 6 cc. of 1 *N* H_2SO_4 by refluxing in a boiling water-bath for 6 hours. When cold, the hydrolysate was diluted with an equal volume of water and nearly neutralized with the saturated $Ba(OH)_2$ (A small quantity of H_2SO_4 was left unprecipitated).

The centrifuged supernatant and the washings of the centrifugate were distilled in vacuo to 0.5 cc. and 0.015 cc. was put on a paper strip. Solvent, *n*-butylacetate-acetic acid-butanol-methanol-water (3:2:2:1:1). 30°C. Solvent front run 45 cm. Developer, aniline-phthalic acid of Partridge. The chromatograms are reproduced diagrammatically in Fig. 1. Either of the preparations contained glucosamine, galactose and mannose.

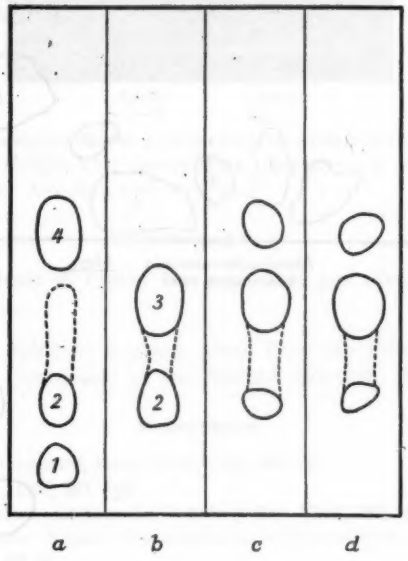


Fig. 1. *n*-Butylacetate-acetic acid-butanol-methanol-water (3:2:2:1:1) chromatograms.
a and b: standards (0.015 cc. of sugar solutions was applied, which were 1% with respect to each individual sugar). c: Ch. I. d: Ch. II. 1: chondrosamine sulfate, 2: glucosamine sulfate, 3: galactose, 4: mannose.

2) Amino acid components (Figs. 2 and 3). 60 mg. of each of the preparations were heated under return condenser with 6 cc. of 6 *N* HCl (in a boiling water-bath, for 16 hours). The hydrolysate was evaporated on a water-bath and the remainder furtheron evaporated with water many times to remove HCl as far as possible. Finally it was taken up in 0.5 cc. of water (the pH of the solution 2.0) and after eliminating the insoluble, 0.1 cc. was applied for irrigation. Solvents: primary run, butanol-acetic acid-water (4:1:5); secondary run, phenol-water (4:1) in NH_3-atmosphere. 20°C. Solvent fronts run 20 cm. Developer, 0.2%

Fig. 2

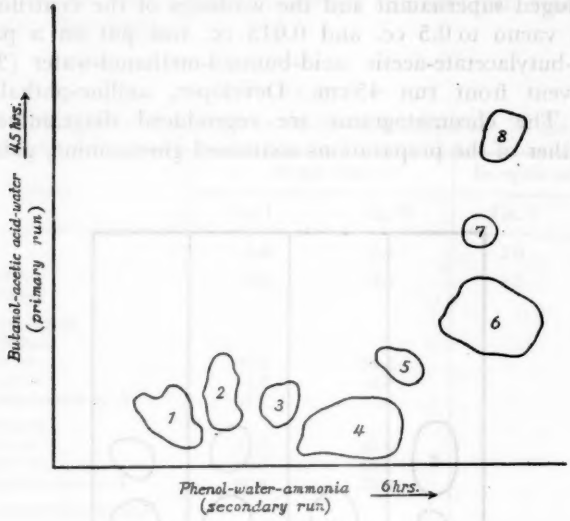


Fig. 3

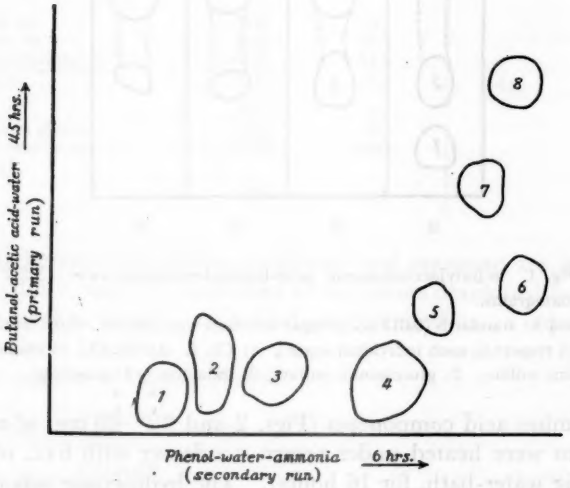


Fig. 2. Butanol-acetic acid-water (4:1:5)/phenol-water (4:1) in NH_3-atmosphere chromatogram of Ch. I.
1: aspartic acid, 2: glutamic acid, 3: glycine, 4: hexosamine, 5: alanine, 6: proline or hydroxyproline, 7: valine or methioninesulfone, 8: leucine or isoleucine.

Fig. 3. Butanol-acetic acid-water (4:1:5)/phenol-water (4:1) in NH_3-atmosphere chromatogram of Ch. II.
1–8 signify the same as in Fig. 2.

ninhydrin in butanol.

In both of the substances, the spots corresponded to aspartic and glutamic acid, glycine, a hexosamine, alanine, proline or hydroxyproline, methioninesulfone or valine and leucine or isoleucine.

Electrophoresis. Ch. I was examined in a Tiselius apparatus. As the pattern (Fig. 4) demonstrates, it was made up of only one component (slow moving).

Fig. 4. An electrophoresis pattern of a 0.6% solution of Ch. I in phosphate buffer, pH 7.8, I=0.29; 8°C; current 10 ma. Exposure 1 h. and 40 min. after starting current. Ascending limb to the left.

Summary

The carbohydrate of casein was separated and investigated regarding composition and else.

This work was aided by a grant, given from the Education Department through the Grant Committee of the Science Council. H. M.

References

1) Sörensen and Haugaard, Bioch. Zeits., 1933, **260**, 247.
2) Sörensen, ibid., 1936, **287**, 140.
3) Haneda, Sinokawa and Yasuoka, Tohoku J. Exp. Med., 1944, **46**, 263.
4) Inoue and Kudo, Nippon Nogeikagaku Kaisi (Journal of the Agricultural Chemical Society of Japan), 1948, **22**, 52.

The Tohoku Journal of Experimental Medicine, Vol. 56, No. 3, 1952

A Modification of Bromsulfalein Test and Its Relation to Liver Biopsy Findings in Children

By

Yuji Sato
(佐藤 雄治)

(From the Department of Pediatrics of Prof. Ts. Arakawa, and the Department of Pathology of Prof. K. Sato, Hirosaki University, Hirosaki)

(Received for publication, March 14, 1952)

As a histopatholgical finding of the liver, 'cloudy swelling' was not uncommonly observed in the biopsy specimens obtained from children with or without nutritional dystrophy.[1)2)] Our experience suggests that the majority of the cases with such a liver change as 'cloudy swelling' used to give a negative result in the bromsulfalein test, in which the dye was injected in an amount of 5 mg. per kg. of body weight.

In this paper we shall report a modified bromsulfalein test and its relation to the liver biopsy findings in both clincal and animal materials.

Experimental

Method and Materials

As a routine clinical practice, the bromsulfalein test seems to be carried out in such a way that the dye is injected intravenously in an amount of 5 mg. per kg. of body weight, and retention of dye in the blood is read at the end of 30 minutes after the injection.

A modified bromsulfalein test:—Modification of the original method consists only in using an increased amount of dye to be injected; bromsulfalein (a preparation from Daiichi Seiyaku Company) is injected intravenously in an amount of *10 mg.* per kg. of body weight and retention of dye in the blood is read by means of a comparator at the end of 30 minutes after the injection.

The children subjected to biopsy and the bromsulfalein test were the patients who visited our Clinic, from whom were excluded those showing more or less retention of bromsulfalein in the blood at the end of 30 minutes after the injection of dye in an amount of 5 mg. per kg. of body weight.

Liver biopsies[3] were carried out on the same day when the bromsulfalein tests were done.

Results and Comment

Clinical observations—According to pathological findings of the liver biopsy, all the cases were classified in the following 3 groups: Group I, including the cases with a mild degree of cloudy swelling of the liver; Group II, including the cases with a moderate degree of cloudy swelling of the liver; and Group III, including the cases with the liver showing both cloudy swelling of moderate degree and portal fibrosis associated with diffuse fibrosis (Cf. Tables I, II and III).

TABLE I

Liver Biopsy Findings and Bromsulfalein Test (10 mg. per kg.) in Group I

No. of biopsy	Age (y.m.)	Infiltration of cells		Changes in cytoplasm			Fibrosis		Clinical diagnosis	B.S.* Ret.%
		Lobules	Portal tracts	Swelling	Fatty granules	Fatty drops	Diffuse	Portal		
99	7.7.	−	±	+	±	−	−	−	Ascariasis	7
71	8.0.	−	−	−	−	−	−	−	Pharyngitis acuta	0
76	3.2.	−	−	+	−	−	−	−	Rickets	0
77	2.9.	−	−	−	+	−	−	−	Angina acuta	0
65	5.6.	−	−	±	−	−	−	−	Pleurisy	0
53	1.3.	−	−	−	−	−	−	−	Measles?	0
55	5.4.	−	−	±	−	−	−	−	Diarrhea	4
48	0.10.	−	+	+	+	−	−	+	Diarrhea	0
34	1.4.	−	−	+	−	−	−	−	Bronchitis	0
39	1.6.	−	−	−	±	−	−	−	Pharyngitis	0
68	3.1.	−	±	±	±	−	−	−	Diarrhea	0
70	5.11.	−	−	+	+	−	−	−	Angina acuta	0
39	1.6.	−	−	+	+	−	−	−	Pharyngitis	0

* Retention of bromsulfalein in the blood was read at the end of 30 minutes after the injection, and the results are expressed in per cent, a concentration of 10 mg. per 100 cc. blood representing 100 per cent.

As already mentioned, the cases in which retention of dye in the blood was observed in the original method of bromsulfalein test were excluded from this investigation. With the modified method of bromsulfalein test, retention of dye was found in 2 cases out of 13 cases of Group I, in 8 cases out of 14 cases of Group II and in 9 cases out of 12 cases of Group III respectively; that is to say, it seems, that the incidence of the cases showing retention of dye was found to be the lowest in Group I,

TABLE II

Liver Biopsy Findings and Bromsulfalein Test (10 mg. per kg.) in Group II

No. of biopsy	Age (y.m.)	Infiltration of cells		Changes in cytoplasm			Fibrosis		Clinical diagnosis	B.S.* Ret. %
		Lobules	Portal tracts	Swelling	Fatty granules	Fatty crops	Diffuse	Portal		
112	3.1.	+	+	++	−	−	−	−	Izumi fever	3
104	4.6.	−	−	++	+	−	−	−	Izumi fever	0
106	7.10.	−	+	++	−	−	−	−	Izumi fever	13
107	4.6.	−	+	++	+	−	−	−	Izumi fever	0
95	4.11.	−	−	++	+	−	−	−	Ascariasis	7
98	1.11.	−	−	+	++	−	−	−	Epidemic vomiting	7
72	9.0.	−	+	++	+	−	−	−	Hepatitis acuta	3
78	6.4.	−	++	+	+++	−	−	−	Tuberculosis of the lungs	40
57	1.8.	−	−	++	+	−	−	±	Heine-Medin's disease	5
50	0.9.	−	++	++	+	−	−	−	Starch injury	0
10	1.8.	−	−	+++	±	−	−	−	Dysentery	5
87	7.0.	+	+	++	++	−	−	−	Izumi fever	0
58	3.10.	−	+	+++	−	−	−	−	Bronchitis	0
37	3.7.	−	±	++	++	−	−	−	Measles	0

TABLE III

Liver Biopsy Findings and Bromsulfalein Test (10 mg. per kg.) in Group III

No. of biopsy	Age (y.m.)	Infiltration of cells		Changes in cytoplasm			Fibrosis		Clinical diagnosis	B.S.* Ret.%
		Lobules	Portal tracts	Swelling	Fatty granules	Fatty drops	Diffuse	Portal		
102	4.7.	−	+	−	+	−	+	++	Congestive hepatomegaly	13
96	2.7.	+	++	+	+	−	+	++	Scarlatina	10
93	1.9.	−	+	+	+	−	+	++	Izumi fever	10
83	11.5.	−	+	±	+	−	±	++	Ascariasis	15
63	4.2.	−	±	+	++	−	+	++	Tuberculosis of the lungs	0
36	2.2.	−	+	+	+	−	+	++	Miliary tuberculosis	10
41	7.10.	−	−	+	++	−	+	++	Tuberculosis of the lungs	0
115	8.4.	−	+	++	+	−	++	++	Tuberculosis of the lungs	5
105	6.0.	−	−	++	+	−	+	++	Hepatomegaly	10
97	0.5.	+	++	+	++	++	++	+++	Miliary tuberculosis	2
40	2.0.	+	+	+	+++	−	++	+++	Ascariasis	0
38	6.0.	+	+	++	++	−	−	++	Hepatomegaly	25

intermediate in Group II and the highest in Group III.

No unfavourable effects were observed on any of the cases subjected to the intravenous injection of bromsulfalein in an amount of 10 mg. per kg. of body weight.

Animal experiments—Liver biopsies and the modified bromsulfalein tests were carried out on 3 rabbits (Nos. 1, 2 and 3) before and after poisoning with carbon tetrachloride as illustrated in Table IV.

TABLE VI

Illustration of Animal Experiment on Rabbits

Date (1951)		No. 1 (2.7 kg.)	No. 2 (2.7 kg.)	No. 3 (2.4 kg.)
18. XII.	B.S.—Ret.%* (10 mg./kg.)	0	0	0
19. XII.		0.05 cc. of C Cl_4 intraperitoneally		
20. XII.	B.S.—Ret.% (10 mg./kg.)	0	0	0
21. XII.		0.05 cc. of C Cl_4 intraperitoneally		
22. XII.	B.S.—Ret.% (10 mg./kg.)	0	2	0
24. XII.		0.05 cc. of C Cl_4 intraperitoneally		
25. XII.	B.S.—Ret.% (10 mg./kg.)	13	30	0
		Liver biopsies were performed		
27. XII.	B.S.—Ret.% (5 mg./kg.)	2	——	0

TABLE V

Liver Biopsy Findings of Rabbits Poisoned with C Cl_4

No. of rabbit / Histopathological findings	No. 1	No. 2	No. 3
Necrosis	+	##	−
Vacuoles	+	##	−
Fatty granules	++	++	+
Dilation of the central veins	+	++	−
Cell infiltration in the portal tracts	##	++	+

On December 25, when retention of dye, in the modified bromsulfalein test, was 15%, 30% and 0% in Nos. 1, 2 and 3 respectively, liver biopsies were carried out and revealed that histopathological alterations of the livers were closely related in their severity to the blood level of dye (Cf. Tables IV and V).

CONCLUSIONS

On the basis of clinical and animal findings, a modification of the

bromsulfalein test (using the dye in an amount of *10 mg.* per kg. of body weight) seemed capable of revealing histopathological alterations of the liver, found on biopsy, which could not be detected by means of the ordinary bromsulfalein test (using the dye in an amount of 5 mg. per kg.)

This work was carried out by a Grant in Aid for Developmental Scientific Research of Ministry of Education.

References

1) Achar, S. T., Brit. Med. J., 1950, No. 4655, 701.
2) Arakawa, Ts., Wada, Sh. and Takahashi, F., Tohoku J. Exp. Med., 1951, **55**, 61.
3) Sato, Y., and Arakawa, Ts., ibid., this volume, p. 59. To be published in a comming issue of this Journal.

The Tohoku Journal of Experimental Medicine, Vol. 56, No. 2, 1952

Macrocytic Anemia and Its Relation to Liver Biopsy of Nutritional Dystrophy in Children. Part I

(Studies on the Nutrition of Children in Hirosaki Area. 12th Report)

By

Shigeho Wada and **Yuji Sato**
(和田 重穂) (佐藤雄治)

(From the Department of Pediatrics of Prof. Ts. Arakawa and the Department of Pathology of Prof. K. Sato, Hirosaki University)

(Received for publication, March 14, 1952)

Introduction

As already reported by Arakawa *et al.*[1] infantile pellagra or nutritional dystrophy[2] has been of endemic occurrence in Hirosaki Area and macrocytic anemia is commonly found in the children with edematous or pellagrous form of nutritional dystrophy.[1][3] In our preceding papers,[1][3] volume index[4] was used as an indicator of macrocytosis. Regarding the relationship between the mean corpuscular volume and mean corpuscular diameter, Whitby and Britton[5] described: "The mean corpuscular volume and mean corpuscular diameter are always increased in approximately parallel amounts in macrocytic anemias, so that an increase in both these factors is an indication for liver therapy. Mean corpuscular volume by itself is a reliable indicator of macrocytosis, with the exception of acholuric jaundice. When there is this phenomenon of spherocytosis, the mean corpuscular volume and mean corpuscular diameter do not preserve their normal relationship; the diameter is relatively reduced and the volume is relatively increased." On the other hand Himsworth[14], described: "Anemia is common in chronic parenchymal failure of the liver. Two types may occur. One is microcytic, or normocytic; the other shows an increased mean cell diameter and color index, and hematocrit value is, however, low so that the cells in this anemia must be abnormally broad and thin. This anemia is similar to that produced experimentally by deficiency of methionine, but it does not react to an excess of dietary protein, liver extracts or iron."

Thus under the term of macrocytic anemia two types may be included; one is anemia with an increased volume index (excepting spherocytosis)

TABLE I

Relation among Clinical Manifestations, Macrocytic Anemia and the Liver Biopsy Finding of Cloudy Swelling in Children with or without Nutritional Dystrophy

Date (1951)	No. of biopsy	Name, age (y.m.) and sex	Infilt. of cells		Changes in cytoplasm				Fibrosis		Red corpuscles			Clinical diagnosis	Manifestations of Avitaminosis B_2
			Lobules	Portal tracts	Swelling	Granular appearance	Fatty granules	Fatty droplets	Diffuse	Portal	Count (millions)	Volume index	Diameter μ (mean)		
10/XII	1	J.T. 3.1. ♀	+	+	++	−	−	−	−	−	3.85	0.99	7.45	Izumi fever	−
20/XI	2	A.T. 4.6. ♂	−	−	++	+	+	−	−	−	3.87	1.08		Izums fever	−
3/XII	3	S.M. 7.10. ♂	−	+	++	−	−	−	−	−	3.99	1.09	7.25	Izumi fever	−
28/XI	4	I.K. 4.6. ♂	−	+	++	+	+	−	−	−	3.94	1.09	7.46	Izumi fever	−
6/XI	5	Y.C. 4.11. ♂	−	−	++	+	+	−	−	−	3.51	1.19		Ascariasis	+
13/XI	6	K.M. 1.11. ♀	−	−	+	+	++	−	−	+	4.08	1.16		Epidemic Vomiting	−
15/XI	7	S.N. 7.7. ♀	−	±	+	+	±	−	−	+	3.60	1.12		Ascariasis	+
15/XI	8	T.E. 5.0. ♂	−	−	−	+	±	−	−	−	2.99	1.20		Izumi fever	+
29/VII	9	S.N. 8.0. ♀	−	−	−	−	−	−	−	−	4.03	1.10		Pharyngitis acuta	−
10/IX	10	F.K. 9.0. ♂	−	+	++	+	+	−	−	−	4.18	1.02	7.76	Infectious hepatitis ?	−
20/IX	11	S.T. 3.2. ♀	−	−	+	+	−	−	−	−	4.18	1.07		Rickets	−
20/IX	12	I.Z. 2.9. ♂	−	−	−	+	+	−	−	−	3.82	1.06		Angina acuta	−
22/IX	13	K.K. 6.4. ♂	−	++	+	+	++	−	−	−	3.62	1.26		Tuberculosis of the lungs	++
16/VIII	14	H.T. 4.2. ♂	−	++	+	+	±	−	−	+	3.60	1.13		Miliary tuberculosis	−
17/VII	15	K.A. 5.6. ♂	−	−	±	±	−	−	−	−	3.41	1.13	7.59	Pleurisy with effusion	+
6/VIII	16	I.H. 1.3. ♂	−	−	−	−	−	−	−	−	4.07	1.09	7.28	Meales	−
9/VIII	17	N.M. 5.4. ♂	−	−	±	±	±	−	−	−	4.18	1.00	7.36	Diarrhea	−
10/VIII	18	M.T. 1.8. ♀	−	−	++	+	+	−	−	±	3.78	1.13		Poliomyelitis anterior acuta	−
1/VIII	19	N.M. 0.10. ♀	−	+	+	+	+	−	−	+	3.91	1.18		Diarrhea	−

29/VI	20	T.K. 1.6.♂	−	−	−	−	±	−	−	−	4.12	1.14	Pharyngitis acuta	−
26/VI	21	U.T. 1.8.♂	−	−	++	++	±	−	−	−	3.99	1.12	Dysentery	−
22/II	22	O.S. 3.0.♂	−	−	++	+	++	−	−	−	3.4	1.25	Ascariasis	++
10/VI	23	T.N. 3.2.♂	+	−	+	±	−	−	−	−	3.55	1.17	Measles (Bronchopneumonia)	++
20/VI	24	S.M. 4.8.♀	−	−	+	±	−	−	−	−	4.43	0.99	Measles	++
25/IX	25	K.K. 12.0.♀	−	−	+	+	+	±	−	−	3.26	1.32	chron. glom. neph.	+
24/X	26	H.H. 7.0.♂	+	+	++	++	++	−	−	−	3.28	1.17	Izumi fever	−
17/VIII	27	T.N. 6.0.♀	−	±	++	+	+	−	−	−	3.75	1.09	Scarlatina	−
20/VIII	28	K.M. 4.3.♂	−	−	+	+	+	−	−	−	3.68	0.99	Abnormal pigmentation?	−
2/VIII	29	I.H. 3.1.♂	−	±	±	±	±	−	−	−	3.45	1.10	Diarrhea ?	−
29/VIII	30	Y.Y. 5.11.♀	−	−	+	+	+	−	−	−	3.65	0.99	Angina acuta	−
15/VIII	31	O.K. 3.10.♂	−	+	++	+	−	−	−	−	3.83	1.16	Bronchitis acuta	−
12/VII	32	T.K. 2.0.♂	−	−	++	+	++	−	−	−	4.20	1.16	Bronchitis acuta	++
16/VII	33	K.S. 2.1.♂	−	±	++	++	++	−	−	−	3.50	1.12	Measles	++
29/VI	34	T.K. 1.6.♀	−	−	+	+	±	−	−	−	4.12	1.14	Pharyngitis acuta	−
28/VI	35	M.M. 5.2.♀	−	−	++	±	±	−	−	−	3.44	1.08	Tuberculosis of the bone	−
22/VI	36	K.M. 2.8.♂	−	+	+	+	+	−	−	±	3.91	1.15	Chron. diarrhea	+
3/II	37	F.K. 3.10.♀	−	−	+	+	++	−	−	−	4.16	1.20	Pertussis	+

and the other is that with an increased mean corpuscular diameter in spite of an unincreased volume index. For the sake of conveninence we shall tentatively call the former macrocytic anemia of Type I and the latter macrocytic anemia of Type II. These two types of macrocytic anemia have been found among the children with nutritional dystrophy in Hirosaki Area. In the present paper we shall report pathological findings of the liver biopsies and their relation to macrocytic anemias in children with or without nutritional dystrophy.

Experimental

Method and Materials

Blood examination and needle biopsy of the liver were carried out

in the same way as described in the preceding papers.[1)3)6)] The measurement of cell diameter was performed by means of a micrometer disk. Of the children who visited our Clinic with complaints other than nutritional dystrophy itself, about half, on close examination, showed some evidences of nutritional dystrophy. All of these children, with and without nutritional dystrophy, were subjected to these examinations.

Results and Comment

The results obtained are given in Tables I, II, III and IV. Pathological findings of the biopsied liver were classified tentatively in the following 3 groups; (1) cloudy swelling, (2) fatty infiltration and (3) portal fibrosis associated with diffuse fibrosis. Such a change as revealed fatty granules stained with Suden III was not included in the group of 'fatty infiltration,' but in the group of 'cloudy swelling' of the liver cells. Pathological diagnosis of 'fatty infiltration' was confined to only the cases in which fatty droplets or drops stained with Sudan III were observed in a considerable amount in the liver parenchyma.

Relation between clinical manifestations of nutritional dystrophy and the occurrence of macrocytic anemia of Type I. As will be seen from Table IV, nutritional dystrophy was found in 43 cases out of 77 examined; out of 43 of nutritional dystrophy, 27 (62.7%) showed macrocytic anemia of Type I, while out of 34 cases without nutritional dystrophy, only 10 (29.4%) showed macrocytic anemia of this type. It may be concluded, therefore, that macrocytic anemia of Type I is more frequently observed in children with nutritional dystrophy than in those without it.

Relation between the pathological changes of the liver and macrocytic anemia of Type I in nutritional dystrophy. As will be seen from Table IV, macrocytic anemia of Type I was found in 12 cases (92.3%) out of 13 with cloudy swelling, in 4 cases (50%) out of 8 with fatty infiltration and in 11 cases (50%) out of 22 with portal fibrosis associated with diffuse fibrosis. The result may suggest that, among the 3 groups of liver lesions revealed by biopsy, cloudy swelling of the liver shows the most close relation to the occurrence of macrocytic anemia of Type I. In our preceding paper[3)] we reported that macroctyic anemia of Type I was commonly found among the children with nutritional dystrophy in Hirosaki Area and that it responded sharply to folic acid but only rarely to vitamin B_{12}. According to Isräls and Sharp,[7)] macrocytic anemia of idiopathic steatorrhea seemed to respond sharply to folic acid but not to vitamin B_{12}, and there was no evidence for suggesting dietary deficiency in folic acid in the patients with idiopathic steatorrhea. We also have no experimental data on folic acid content in food stuffs with which the children with nutritional dystrophy had been provided. But it may be supposed that

their diet was deficient in folic acid, because their dietetic history revealed very few green vegetables, and very little liver, corn and beef, which[8] contain folic acid in a considerable quantity. Respecting pathogenesis of macrocytic anemia in infancy May *et al.*[9] stated that chronic vitamin C deficiency would be the primary cause, by which metabolism of folic acid might be disturbed secondarily. Nichol and Welch[10] reported that addition of folic acid to slices of normal liver increased the amounts of citrovorum factor, the yield being further increased by the addition of certain reducing agents, especially ascorbic acid. Folinic acid preparation promotes blood regeneration in persons with pernicious anemia, nutritional macrocytic and tropical sprue in relapse.[11] If folic acid plays a role in pathogenesis of macrocytic anemia of Type I, it may be supposed from the investigations above quoted that utilization of folic acid (its conversion to folinic acid) would be more severely disturbed in the liver with cloudy swelling than in those with fatty infiltration or portal fibrosis associated with diffuse fibrosis.

Relation between macrocytic anemia of Type II and pathological findings of the liver biopsies. Macrocytic anemia of Type II was found in 4 cases (Cases 1, 2, 3, and 16) out of 7 (1, 2, 3, 9, 15, 16 and 17) with the liver showing portal fibrosis associated with diffuse fibrosis (Cf. Table III); in two cases (Cases 1 and 3) out of 3 (Cases 1, 3 and 8) with fatty infiltration (Cf. Table II); and in none out of 7 cases (Cases 1, 3, 4, 10, 15, 16, and 17) with cloudy swelling of the liver (Cf. Table I).

Macrocytic anemia of Type II is that showing an increased mean corpuscular diameter but not increased hematocrit value, and this type of anemia was said[14] to be encountered in association with the chronic parenchymal failure of the liver.

Out of 14 cases with fatty infiltration of the liver, 8 (Cases 3, 4, 5, 9, 11, 12, 13, and 14 in Table II) were those associated with diarrhea of a considerable duration and nourished on a diet restricted to a little polished rice gruel; that is, these cases may be assumed to be those of 'acute protein deficiency[12]' occurring in addition to the background of nutritional dystrophy. Fatty infiltration observed in the other 6 cases of this group (Cf. Table II) may be assumed to be a consequence of chronic protein deficiency. According to Himsworth,[15] (1) prolonged fatty infiltration of the liver caused portal fibrosis with diffuse fibrosis of this organ; further, (2) this fatty infiltration might be the result of dietary deficiency in animal protein. Portal fibrosis associated with diffuse fibrosis, found in at least 16 cases out of 25 shown in Table III (Cases 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 16, 17, 18, 21 and 22) may be explained by the Himsworth theory cited above. According to Himsworth's conception, fatty infiltration as well as portal fibrosis associated with diffuse

TABLE II

Relation among Clinical Diagnosis, Anemia and Liver Biopsy Finding of Fatty Infiltration in Children with or without Nutritional Dystrophy

Date (1951)	No. of biopsy	Name, age (m.m.) and sex	Infilt. of cells		Changes in cytoplasm					Fibrosis		Red corpuscles			Clinical diagnosis	Manifestations of Avitaminosis B_2
			Lobulus	Portal tracts	Swelling	Granular appearance	Fatty granules	Fatty droplets	Fatty drops	Diffuse	Portal	Count (millions)	Volume index	Diameter μ (mean)		
6/XII	1	S.N. 4.0.♀	+	+	+	−	−	++	+++	−	−	3.5	1.0	8	Ascariasis	−
24/X	2	I.O. 2.0.♂	+	+	+	−	−	++	++	−	−	3.89	1.19		Starch injury	−
29/VIII	3	K.E. 2.1·♀	±	+	+	−	−	++	+++			2.16	1.83	8.44	Dysentery	+
30/VII	4	K.Y. 1.10.♂	−	−	+	−	−	++	++	−	−	3.51	1.10		Dysentery	+
13/VII	5	K.H. 2.2.♀	+	+	+	+	+++	+	−	−	−	4.14	1.06		Measles	+
28/VI	6	S.Y. 2.0.♂	−	−	+	+	++	+	−	−	+	4.80	0.82		Rickets	+
29/VI	7	N.K. 2.1.♀	−	±	+	++	+++	++	−	−	+	4.41	0.92		Ascariasis	−
22/VI	8	M.Y. 6.7.♀	−	+	+	−	−	++	+++	−	−	3.04	1.08	7.17	Miliary tuberclosis	−
22/VI	9	N.K. 3.7.♂	−	+	++	+	++	+++	++	−	−	3.93	1.2		Measles	++
25/VI	10	S.K. 1.4.♀	−	−	++	+	+	+	+	±	+	3.71	1.04		Rickets	−
26/VI	11	K.T. 2.4.♀	−	+	++	+	++	++	−	−	+	3.91	1.07		Dysentery (Starch injury)	−
15/VI	12	H.S. 1.4.♂	−	−	++	+	+++	++	−	−	−	3.32	1.20		Dysentery	+
27/VI	13	T.M. 1.2.♀	+	−	+	+	+++	+	−	−	−	3.29	1.10		Nutritional edema	+
11/VI	14	H.8. 1.4.♂	−	→	+	+	++	++	++	−	−	3.32	1.20		Dysentery	+

fibrosis were in a series of pathologic changes resulting from deficiency in animal protein or lipotropic facotrs. Macrocytic anemia of Type II was more frequently found in the cases with fatty infiltration or portal fibrosis associated with diffuse fibrosis than in those with cloudy swelling of the liver; while the incidence of macrocytic anemia of Type I was decidedly higher in the cases with cloudy swelling than in those with the other pathological findings of the liver biopsies.

TABLE III

Relation among Clinical Diagnosis, Macrocytic Anemia and the Liver Biopsy of Portal Fibrosis associated with Diffuse Fibrosis in Children with or without Nutritional Dystrophy

Date (1951)	No. of biopsy	Name, age (y.m.) and sex	Infilt. of cells		Changes in cytoplasm			Fibrosis		Red corpuscles			Clinical diagnosis	Manifestations of Avitaminosis B_2
			Lobulus	Portal tracts	Swelling	Granular appearance	Fatty granules	Fatty droplets	Diffuse	Portal	Count (millions)	Volume index	Diameter μ (mean)		
17/XII	1	Y.Y. 8.4.♀	−	+	++	+	+	−	+++	++	4.31	0.96	8.18	Tuberculosis of the lungs	+
11/XII	2	Y.N. 3.1.♂	+	+	++	+	++	−	++	++	4.12	1.08	8.36	Hepatomegaly	+
30/IX	3	J.K. 6.0.♂	−	−	++	+	+	−	+	++	3.23	1.22	8.04	Hepatomegaly	+
16/XI	4	S.S. 4.7.♂	−	+	−	+	+	−	+	++	3.00	1.25	8.36	Congestive hepatomegaly	+
9/XI	5	S.A. 0.5.♂	+	++	+	++	++	++	++	+++	3.15	1.26		Miliary tuberculosis	+
7/XI	6	I.K. 2.7.♂	+	++	+	+	+	−	+	++	3.94	1.26		Scarlatina	+
6/XI	7	T.M. 1.9.♂	−	+	+	+	+	−	+	++	3.05	1.30		Izumi fever	++
31/X	8	F.R. 1.1.♀	−	+	±	+	++	−	+	++	3.78	1.23		Ascariasis	+++
28/IX	9	S.F. 11.5.♀	−	+	±	+	+	−	±	++	4.27	1.04	7.98	Ascariasis	+++
15/IX	10	K.K. 4.11.♀	−	±	±	+	+	−	+	+	3.51	1.22		Tuberculosis of the lungs	+++
15/IX	11	S.K. 2.3.♀	−	±	+	+	+++	+	±	+	3.95	1.13		Izumi fever	+++
17/VIII	12	S.K. 4.2.♀	−	±	+	+	++	±	+	++	3.54	1.15		Tuberculosis of the lungs	++
2/VIII	13	M.S. 1.2.♂	−	++	+	+	++	++	+	+++	3.15	1.47		Tetany and rickets	+++
1/VIII	14	S.K. 5.0.♂	+	+	+	+	++	+	+	++	4.35	0.84	7.71	Scarlatina	+
31/VII	15	O.T. 1.4.♂	+	+	+	+	+++	+	±	++	4.76	0.86	7.45	Ariboflavinosis	+++
18/VII	16	S.K. 2.0.♂	+	+	+	+	+++	−	++	+++	4.11	0.84	8.05	Ascariasis	+++
17/VI	17	K.S. 6.0.♀	+	+	++	+	++	−	+	++	3.51	0.9	7.46	Hepatomegaly	+
26/VI	18	O.H. 2.8.♂	−	++	+	+	++	+++	++	+++	4.25	1.03		Pellagra	+++
18/VIII	19	K.K. 1.6.♂	−	−	+	+	±	−	+	++	3.91	1.06		Diarrhea followed by measles	−
16/VII	20	O.M. 2.2.♂	−	+	+	+	+	−	+	++	3.95	0.88		Miliary tuberculosis	+++
18/VI	21	U.K. 1.3.♂	+	+	++	±	−	−	+	++	3.34	1.02		Rickets	−
25/X	22	M.K. 3.4.♂	−	−	+	+	++	+	−	+	3.97	1.10		Miliary tuberculosis	+
20/VII	23	S.H. 7.10.♂	−	−	+	+	++	+	+	++	4.05	0.85		Tuberculosis of the lungs	+
16/VI	24	K.T. 5.8.♂	−	+	++	+	+++	−	±	+	2.80	1.22		Dysentery	+++
21/VI	25	K.C. 6.7.♀	±	±	++	+	±	−	−	+	3.81	1.08		Infectious hepatitis	−

TABLE IV

Relation between Pathological Changes of the Biopsied Liver and Macrocytic Anemia of Type I in Children with or without Nutritional Dystrophy

Pathological changes of biopsied liver / Anemia	Volume index <1.11		Volume index ≦1.10			
Portal fibrosis associated with diffuse fibrosis	11*	(0)†	11	(3)	22	(3)
Fatty infiltration	4	(1)	4	(5)	8	(6)
Cloudy swelling	12	(9)	1	(15)	13	(24)
	27	(10)	6	(23)	43	(34)

* 11; Number of cases with nutritional dystrophy.
† (0); Number of cases without nutritional dystrophy.

Animal experimentations respecting the relation between macrocytic anemias of both Type I and Type II and liver biopsy findings will be reported in this Journal in the future.

Relation between clinical manifestations of nutritional dystrophy and pathological changes of the biopsied liver. Clinical manifestations of nutritional dystrophy, especially angular stomatitis, cheilosis, hyperpigmentation of the skin, edema and atrophy or mushroomlike appearance of the lingual papillae were found in 22 cases (88.0%) out of 25 cases with the liver of portal fibrosis associated with diffuse fibrosis; in 8 cases (57.1%) out of 14 cases with the liver of fatty infiltration; and in 13 cases (35.1%) out of 37 cases in which cloudy swelling was revealed in the biopsied liver. Lichtman[13] presumed that niacin must be methylated in the body, so if niacin is utilized in a large dose in a body, the requirement for methionine or protein should be increased; and that riboflavin storage in the liver might also require increased amounts of methionine or protein. Himsworth[15] stated that portal fibrosis associated with diffuse fibrosis of the liver was accepted as resulting from prolonged fatty infiltration of the liver, consequently from chronic deficiency in protein or methionine. From these reports above quoted, it may be clearly seen that the higher incidence of B_2-avitaminotic signs was found in the cases with the liver showing portal fibrosis associated with diffuse fibrosis than in those with the liver showing fatty infiltration or cloudy swelling.

Summary and Conclusions

On 77 cases of children with or without nutritional dystrophy, blood

examination and needle biopsy of the liver were carried out. Pathological findings of the liver biopsies were classified in the 3 groups: cloudy swelling, fatty infiltration and portal fibrosis associated with diffuse fibrosis.

Two types of macrocytic anemia were found among these children with nutritional dystrophy: one showed an increased volume index (macrocytic anemia of Type I) and the other an increased mean corpuscular diameter in spite of no increase in volume index (macrocytic anemia of Type II). Macrocytic anemia of Type I was more frequently found in the cases with the liver changes of cloudy swelling than in those with the liver lesions such as fatty infiltration or portal fibrosis associated with diffuse fibrosis; while macrocytic anemia of Type II was more frequently found in the cases with the liver showing portal fibrosis associated with diffuse fibrosis than in those with cloudy swelling alone.

Portal fibrosis with diffuse fibrosis of the liver (at least in 16 cases out of 25 cases) was considered as a consequence of prolonged fatty infiltration of this organ, accordingly of chronic deficiency in protein or methionine.

Clinial manifestations of B_2-avitaminosis were observed most conspicuously in their frequency as well as in their intensity in the cases with portal fibrosis with diffuse fibrosis as compared with the cases showing the other changes of the biopsied liver.

This work was carried out by a Grant in Aid for Developmental Scientific Research of Ministry of Education. Prof. Ts. Arakawa.

References

1) Arakawa, Ts., Wada, Sh. and Takahashi, F., Tohoku J. Exp. Med., 1951, **55**, 61.

2) Achar, S. T., Brit. Med. J., 1950, No. 4655, 701.

3) Wada, Sh. and Arakawa, Ts., Blood Picture of Nutrional Dystrophy in Children; Effect of Folic Acid and Vitamin B_{12} upon It. To be published in a coming issue of this Journal.

4) Wintrobe, M. M., Clinical Hematology, 2nd ed., Lea & Febiger, Philadelphia, 1949, 246.

5) Whitby, L.E.H. and Britton, C.J.C., Disorders of the Blood, 5th ed., J. & A. Churchill, London, 1947, 61.

6) Sato, Y. and Arakawa, Ts., Hepatomegalic Liver in Children with Chronic Miliary Tuberculosis and Liver Biopsy Findings. To be published in a coming issue of this Journal.

7) Cited from the Editorial, Lancet, 1950, No. 6608, 767.

8) Sargent, F., New Eng. J. Med., 1947, **237**, 667.

9) May, C. D., Nelson, E. N., Lowe, C. U., and Salmon, R. J., Am. J. Dis. Child., 1950, **80**, 191.

10) Nichol, C. A., and Welch, A. D., Proc. Soc. Exp. Biol. and Med., 1950, **74**, 52; 1950, **74**, 403.

11) Cited from J. A. M. A., 1951, **146**, 733.

12) Arakawa, Ts. and Sato, Y., Acute Enlargement of the Liver in Dysentery or Measles. To be published in a coming issue of this Journal.

13) Lichtman, S.S., Diseases of Liver, Gallbladder and Bile Ducts, 2nd ed., Philadelphia, 1949.

14) Himsworth, H. P., Lectures on the Liver and its Diseases, 2nd ed., Blackwell, Oxford, 1950, 142.

15) Himsworth (14)), pages 82 and 93.

The Tohoku Journal of Experimental Medicine, Vol. 56, No. 3, 1952

Biochemical Studies on Carbohydrates

CLVIII. On the Placental Substances Giving the Skin Reaction of Cancer and Pregnancy

Third Paper

By

Hajime Masamune and **Masayosi Ogawa**

(正宗 一) (小川 政良)

(From the Medico-chemical Institute and the Surgical Clinic of Prof. M. Muto, Tohoku University, Sendai)

(Received for publication, March 14, 1952)

The crude carbohydrate fraction in the First Paper[1] was devoid of the potency to give the skin reaction of cancer and pregnancy. Hence with the aim of chemical study alone, it was freed from proteins and nucleic acids. Unexpectedly, however, the purified fraction, which proved to be a mixture of an itin or more probably an itinsulfuric acid and a hexuronic acid-free polysaccharide coupled with a small grouping of amino acids, showed that biological reaction.

Experiments and Comments

Purification of the Carbohydrate Fraction

1) 6 g. lots of the crude carbohydrate, obtained as described in the First Paper,[1] were taken up in 27 cc. of water and 10% NaOH added carefully to a pH of 7.4. The centrifuged supernatant was yellowish-brownish colored and translucent. The centrifugate was exhausted with small changes of water. The successive solutions were combined (170 cc. in total), acidified with acetic acid (30%) to a pH of 4.5, whereby turbidity increased, and after addition of about 50 mg. sodium acetate (anhydrous), stood overnight. Centrifuged. The nearly clear supernatant here and the washings of the centrifugate (a dilute acetic acid of the same pH was used) were neutralized with NaOH and distilled in vacuo. To the syrupy condensate were added 40 cc. of abs. alcohol and the precipitate washed with abs. alcohol and dried. 2.95 g. of a yellowish non-sticky powder containing phosphorus were given. Biuret positive.

2) 2.85 g. of the substance given were mixed with 1 cc. of water into a paste and 30 cc. of glacial acetic acid (98%) added in drops, with kneading, at first and afterwards in larger portions. The centrifuged supernatant was brownish-tinged and clear. The centrifugate was washed

with 5 cc. portions of the glacial acetic acid until a renewed washing remained uncolored. It was further washed by agitating with a 40 cc. portion of abs. alcohol, covering under 40 cc. of abs. alcohol overnight and again agitating with two 40 cc. portions of abs. alcohol in sequence. After drying, 2.2 g. of a phosphorus-free product were yielded, which was still biuret-positive.

3) It was extracted with 20 cc. of 5% trichloroacetic acid. After repeated centrifugations the supernatant became almost clear (light yellow, a bit viscid). The centrifugate was treated with another changes of 5% trichloroacetic acid and the solutions (in total 44 cc.)were precipitated with dry acetone (250 cc. were requested), washed with abs. alcohol thoroughly and dried. A white powder weighing 800 mg. was yielded.

4) The operation in 2) was applied again.

5) The operation in 3) was processed again. The solutions in 5% trichloroacetic acid here were non-opalescent. The yield, 500 mg.

6) The product was taken up in 20 cc. of water and neutralized and a small insoluble part centrifuged off. The clear supernatant was distilled in vacuo to a syrup and 50 cc. of glacial acetic acid (98%) were added. The precipitate, separated by centrifugation, was washed twice with the glacial acetic acid and four times with abs. alcohol and dried. The amount of substance diminished to 480 mg. The positive biuret reaction was shown, though faint.

Another similar precipitation reduced the substance even to 400 mg., but the biuret test resulted alike, so the writers were compelled to resort to the copper-alkali method[2] which appeared too drastic.

7) It was dissolved in 20 cc. of water (A clear solution of pH 3.0 formed), neutralized with 10% NaOH (3 drops were used. No precipitation happened), and 4 cc. of 10% NaOH and 4 cc. of 12.5% cupric sulfate were added in turn, under violent agitation. Centrifuged. The red-violet supernatant was set aside, and the centrifugate was washed with three 5 cc. portions of 2% NaOH and one 10 cc. portion of 1% NaOH. The final washing remained uncolored. The washings were also set aside. In the next, the washed matter was dissolved with 4 cc. of 10% HCl (The pH of the solution 1.5), precipitated with 50 cc. of abs. alcohol and 10 cc. of the saturated NaCl in abs. alcohol. The centrifugate here was stirred up in 50 cc. of abs. alcohol containing 5 drops of 2% HCl and then washed twice with 50 cc. abs. alcohol, followed by drying. 115 mg. of an almost biuret-negative powder were given.

The mother fluid and washings of the precipitate by $CuSO_4$ and NaOH were acidulated (to pH 6.0) with 10% HCl and distilled to dryness under low pressure. The remainder, well washed with abs. alcohol and dried, was also precipitated from water with $CuSO_4$ and NaOH and

washed. 114 mg. of a substance resembling the one above were produced.

The both products were united and a like procedure effected once more, and 147 mg. of a perfectly biuret-negative, non-colored, non-sticky powder were given. This was hygroscopic and readily soluble in water.

Properties of the Purified Carbohydrate Specimen

Chemical qualitative tests. Biuret-negative. Strongly Molisch-positive. The positive aniline acetate (for pentose and hexuronic acid)- and Neuberg-Saneyoshi (for hexuronic acid)-reaction were shown, though not intensive. Rosenthaler (for methylpentose) as well as the direct and indirect Osaki-Turumi (for hexosamine) resulted in slightly positive. Hydrolysable sulfur detected. Glycogen absent (iodine test).

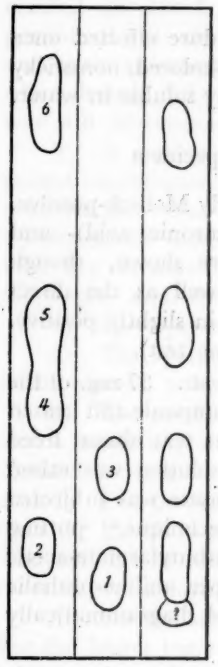
Paper partition chromatography of the sugar components. 37 mg. of the substance were sealed with 3.7 cc. of 1 *N* H_2SO_4 in an ampoule and heated in a boiling water-bath for 5 hours. The hydrolysate was almost freed from H_2SO_4 with the saturated $Ba(OH)_2$ (pH of the solution was raised to 5.8) and distilled in vacuo to 0.8 cc. The condensate was subjected to the chromatography according to Yosizawa's technique,[3] putting 0.01 cc. of it on a paper strip. Solvent mixture, *n*-butylacetate-acetic acid-water (3:2:1). Solvent front run 40 cm. Developer, aniline-phthalic acid of Partridge.[4] The chromatogram is reproduced diagrammatically in Fig. 1 together with the standards.

The four spots correspond to the positions of glucosamine, a hexuronic acid (By the present solvent glucuronic and galacturonic acid can not be well separated), mannose and L-fucose. Besides those an unknown spot marked with ? of less mobility than that of chondrosamine is seen. It is likely that this represents the residue of the unit disaccharide of a probable itinsulfuric acid whose complete hydrolysis is analogized to be difficult.

As mentioned by Masamune,[5] the simultaneous presence of hexose and hexuronic acid in one hexosamine-containing polysaccharide is inconceivable and suggests two kinds of polysaccharide in the preparation.

Chemical quantitative analysis. N (micro Kjeldahl) 2.4%; glucosamine 14.9%; non-amino sugars as mannose: 50.0% by the orcinol method, 52.7% by the indole method, 21.8% by the diphenylamine method I; ash (micro Pregl) 9.8%; ferricyanide reduction after hydrolysis as glucose, 67.6%; iodine use (Macleod and Robison) as glucose 3.9%. To determine the total reduction of the hydrolysable sugars, a weighed amount of the substance was heated at 100°C in an ampoule with 0.5 or 1 *N* H_2SO_4 for a various duration of time. And an aliquot of the hydrolysates

Fig. 1.



were neutralized with 1 *N* KOH, made up to 14 cc. with water, and after addition of 4 cc. of 0.005 *N* ferricyanide solution of Fujita and Iwatake,[6] applied to the procedure of these authors (Cf. also Masamune and Tanabe[7]). The maximum reductions were brought about by the hydrolysates obtained by 7 and 4 hours' heating respectively with 0.5 and 1 *N* H_2SO_4. As expressed as glucose those reductions reached 63.0% in the former case and 67.6% in the latter.

Optical rotation. $[\alpha]_D^6 = \frac{+0.23 \times 100}{0.99 \times 1} = +23.2°$

(in water).

Electrophoretic analysis. It was effected in a Tiselius apparatus as dissolved in phosphate buffer, pH 7.8. The pattern (Fig. 2) demonstrated two

Fig. 2.

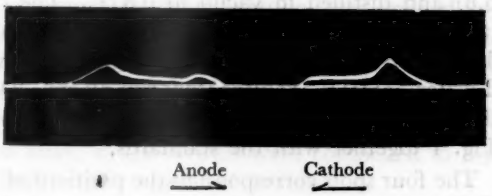


Fig. 1. *n*-Butylacetate-acetic acid-water (3:2:1) chromatogram; duration of test 13.5 hrs., temperature 19±1°C.
a and b: standards, 1: chondrosamine sulfate, 2: glucosamine sulfate, 3: glucuronic acid, 4: galactose, 5: mannose, 6: rhamnose.

Fig. 2. An electrophoretic pattern of a 1% solution of the purified carbohydrate specimen in phosphate buffer, pH 7.8, I=0.29; 10°C; current 16 ma. Exposure 40 minutes after starting current. Ascending limb to the left.

components, as had been expected from the paper-chromatographic result above.

Cancer skin test. Technique, the same as that recorded in the First Paper.[1] The substance showed fair distinction in the largeness of the evolving skin redness between cancerous and non-cancerous patients.

Dilution of substance	Patients	Number of patients tested	Number of positive cases (discr. width, 23mm.)
1:1,000	Canc.	13	11
	Non-canc.	14	5

1:5,000	Canc.	9	8
	Non-canc.	10	1
1:10,000	Canc.	9	4
	Non-canc.	6	1

Summary

A carbohydrate preparation which had the potency of giving cancer skin reaction was separated from placentae. And its chemical and physical properties were investigated. Electrophoresis exhibited two boundaries. The sugars involved were glucosamine, chondrosamine, mannose, a hexuronic acid and L-fucose. By these findings together with Masamune's claim was suggested a mixture of two kinds of hexosamine-containing polysaccharide, one a hexuronic acid-containing and the other a hexuronic acid non-containing.

The Education Department gave a grant through the Grant Committee for Scientific Researches in aid to us. H. Masamune.

References

1) Oh-Uti, Ogawa, Yabuki and Kawasaki, Tohoku J. Exp. Med., this volume, p. 177.
2) Cf. f. i. Satoh, ibid., 1949, **51**, 275.
3) Yosizawa, ibid., 1950, **53**, 128.
4) Partridge, Nature, 1949, **164**, 443.
5) Masamune, ibid., 1949, **50**, 107.
6) Fujita and Iwatake, Bioch. Zeits., 1931, **242**, 43.
7) Masamune and Tanabe, J. Bioch., 1938, **28**, 19.

The Tohoku Journal of Experimental Medicine, Vol. 56, No. 3, 1952

A Method of Simultaneous Estimation of Effective Renal Blood Flow and Circulating Blood Volume by Sodium Para-aminohippurate

By

Takashi Kishine, Kietsu Igarashi, Hideo Tsukamoto,
(岸 根 隆) (五十嵐 喜悦) (塚本 榮夫)
Ko Suto and **Yoshimichi Watanabe**
(須 藤 好) (渡邊 睦道)

(From the Medical Clinic of Prof. F. Nakazawa,
Faculty of Medicine, Tohoku University, Sendai)

(Received for publication, March 20, 1952)

It is well known, that since Smith *et al.*[1] established the estimation of effective renal blood flow (we call it only 'renal blood flow' in this report) by sodium para-aminohippurate (PAH), it has become popular as one of the important methods estimating kidney functions. But by PAH we have found a method estimating not only renal blood flow but also circulating blood volume at the same time, so we are going to report it here.

Theoretical Consideration

According to Chasis *et al.*[2] PAH is impermeable into erythrocytes and PAH in blood is limitted only within plasma, consequently its distribution in the human body concerns only plasma and tissues.

Let Vp be circulating plasma volume.

Let 'a' be PAH quantity in circulating plasma at a certain point of time (we make it 'the start point of time'), when the equilibrium of PAH exists between tissues (including plasma except circulating plasma) and circulating plasma.

Let X be PAH quantity lost in the urine within time t after the start point of time. Let Y be the volume of PAH moved from tissues into circulating plasma within time t.

Let Z be the quantity of PAH reduced in circulating plasma within time t. And we obtain

$$Z=X-Y \quad \ldots\ldots(1)$$

Let dZ be the PAH quantity lost from circulating plasma within very little time dt, and let dX and dY be respectively the PAH quantity lost into urine and the PAH quantity moved from tissues into circulating

plasma within time dt. Then

$$dZ=dX(1-\frac{dY}{dX}) \quad \text{.....(2)}$$

According to Warren *et al.*[3] 88% of PAH is lost into urine while it circulates kidneys once, so if v denotes plasma volume circulating kidneys in one minute, that is, effective renal plasma flow, dX the PAH quantity lost in urine in time dt is

$$dX=\frac{0.88v}{Vp}(a-Z)\ dt \quad \text{.....(3)}$$

From (2) and (3)

$$\frac{dZ}{a-Z}=\frac{0.88v}{Vp}\ (1-\frac{dY}{dX})dt$$

Integrating both sides,

$$-\ln\ (a-Z)=\frac{0.88v}{Vp}(t-\int\frac{dY}{dX}\ dt)$$

If c denotes PAH concentration in circulating plasma when PAH quantity in it is a−Z, we get $c=\frac{a-Z}{Vp}$,

Therefore $-\ln c=\frac{0.88v}{Vp}\ (t-\int\frac{dY}{dX}dt)+\ln\ Vp$.....(4)

However, according to the preceding reports and to our own experiment which we will state afterwards, when equilibrium of PAH is found between circulating plasma and tissues, the logarithm of concentration of it in circulating plasma falls down almost with linear relation to the time t, and so $\int\frac{dY}{dX}\ dt$ in (4) must be linear function of t, but since $\frac{dY}{dX}\neq O$ is evident, $\frac{dY}{dX}=K$ (constant).

Moreover as Y=O when X=O

$$Y=KX \quad \text{.....(5)}$$

Therefore (4) becomes as follows,

$$-\ln\ c=\frac{0.88v\ (1-K)\ t}{Vp}+C\ \text{(C means constant of integration)}$$

If $c=C_o$, when t=o, then C=−ln c, and so

$$\ln c=\ln c_o-\frac{0.88v\ (1-K)}{Vp}t$$

Hence

$$\log c=\log c_o-\frac{0.88v\ (1-K)}{2.303Vp}t \quad \text{.....(6)}$$

This is the formula which shows the relation between the concentration logarithm of PAH in circulating plasma and the time. (It is a straight

line in this case). From this we can get

$$\frac{v(1-K)}{Vp}=\frac{2.617}{t}\log\frac{c_o}{c} \quad \cdots\cdots(7)$$

When PAH quantity in circulating plasma is a and a−Z, the concentration is respectively c_o and c, so $Z=(c_o-c)\,Vp$, and moreover from (1) and (5) $Z=(1-K)\,X$, therefore

$$\frac{1-K}{Vp}=\frac{c_o-c}{X} \quad \cdots\cdots(8)$$

v can be calculated from (7) and (8).

Since $Y=KX$ and $Z=(1-K)\,X$, the ratio of PAH quantity lost from circulating plasma and tissues during the same time is (1−K): K in PAH equilibrium: that is, in the equilibrium of PAH, the ratio of PAH quantity in circulating plasma and tissues relating to the equilibrium is (1−K): K. Hence when PAH quantity in circulating plasma is a−Z, the whole quantity of PAH in tissues is

$$\frac{K}{1-K}(a-Z)+b$$

Here b is the quantity of PAH in the tissues without any relation to PAH equilibrium between circulating plasma and tissues, that is, b is the quantity of PAH in the tissues, which can not move into circulating plasma in spite of the reduction of its concentration in the latter. Suppose the injected quantity is 2000 (mg) and let X be the excreted quantity into urine, and we obtain

$$a-Z+\frac{K}{1-K}(a-Z)+b=2000-X$$

Therefore

$$a-Z=(1-K)\ (2000-X-b)$$

Since $a-Z=c\,Vp$, we obtain

$$\frac{1-K}{Vp}=\frac{c}{2000-X-b} \quad \cdots\cdots(9)$$

$\frac{1-K}{Vp}$ is calculated from (8) and as c and X can be estimated, b can be calculated from (9).

Again from (9)

$$1-K=\frac{cVp}{2000-X-b}=\frac{\frac{cVp}{2000-X}}{1-\frac{b}{2000-X}} \quad \cdots\cdots(10)$$

However, estimating circulating plasma volume by the method of Gibson *et al.*[4] and estimating c and X about 30 minutes after PAH injection, that is c_1 and X_1, we found $\frac{c_1Vp}{2000-X}$ almost constant (0.153), and so we

can calculate 1−K from (10): consequently Vp can be calculated from (8). (Of course Vp can be calculated from $\frac{c_1 Vp}{2000-X_1}=0.153$, and K can be calculated from (8)).

Experiments

We made our experiment about 15 healthy persons out of the faculty of our medical office, who had no past history of cardiovascular diseases or kidney troubles.

Two hours and a half after breakfast without tea, coffee, or tobacco we took their blood to estimate hematocrit readings and instantly after it made them drink 500—700 cc water and made them lie quiet. After 30 minutes, diluting 10 cc of 20% solution of PAH (Daiichi Pure Chemicals Co., LTD) that is 2000 mg of PAH, with 40 cc physiological salt solution, we injected it into their antecubital veins almost in 2 minutes, and according to one of the three methods in the illustration (Fig. 1), we took

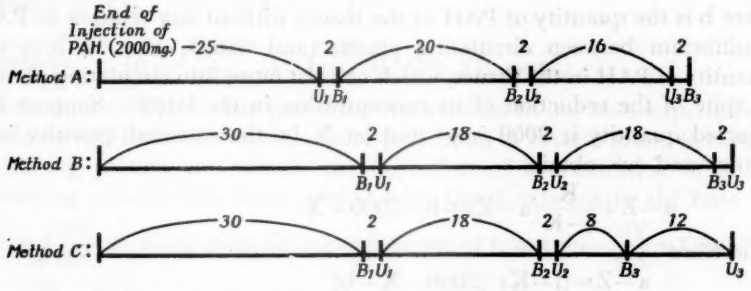


Fig. 1. Patterns of interval planned in our experiment.

blood and urine samples in the exactness of time within 30 seconds. The volume of blood taken was 4 cc each time and dioxalate was used as anticoagulant. The urine was taken spontaneously, but as completely as possible. Instantly after it circulating plasma volume was estimated by the method of Gibson *et al.*[4] PAH concentration was estimated as follows: plasma was cleared of proteins by tri-chloroacetic acid at the proper dilution of plasma, and after the diazotization of the filtrate it was coloured with Tsuda's reagent and colorimetry was done with Pulfrich's photometer (Filter 53). PAH concentration in the urine was also estimated concerning diluted urine, and the whole quantity (X_1, X_2, X_3) of PAH in each fraction of urine (U_1, U_2, U_3) was calculated.

If a coordinate is made from the logarithm of PAH concentration in

plasma and the time, those three points are almost on a straight line. (Those cases where the three points were not on a straight line were repudiated as they were not in good conditions for examination). Accordingly if the straight line is made by the method of the least squares, PAH concentration in circulating plasma at a optional point of time can be found: consequently $\frac{v\,(1-K)}{Vp}$ can be calculated from (7). According to Smith *et al.*[5] nearly 2 minutes are needed for urine produced in kidneys to reach bladder, so if X in (8) denotes PAH quantity in urine from point of time t_1 to t_2, $\frac{1-K}{Vp}$ can be calculated by regarding c_o and c as PAH concentration in circulating plasma respectively 2 minutes before t_1 and t_2. Consequently v can be found from (7). As $\frac{1-K}{Vp}$ can be obtained from either U_2 or U_3, if they are denoted by $\left(\frac{1-K}{Vp}\right)_1$, and $\left(\frac{1-K}{Vp}\right)_2$, the two values v_1, v_2: b_1, b_2 can be obtained concerning v and b. Suppose X_1 is PAH quantity in urine fraction U_1, and c_1 is PAH concentration in circulating plasma corresponding to the time of excretion of U_1, then $\frac{c_1\,Vp}{2000-X_1}=0.153$ and so 1—K, and then K (let it be K_1) can be obtained from (9). Consequently Vp can be obtained from (8). If this Vp will be substituted in (8) concerning the second urine fraction U_2, K concerning U_2 that is K_2 can be obtained. Moreover we estimated effective renal plasma flow (RPF) by the method hitherto used, but then following Alving[6] we used $\frac{c_o-c}{2{,}303\,\log\frac{c_o}{c}}$ as averaged concentration of PAH in plasma. Accordingly we obtained effective renal plasma flow by dividing averaged excretion quantity of PAH in one minute by $0.88\,\frac{c_o-c}{2.303\,\log\frac{c_o}{c}}$. We obtained effective renal blood flow (RBF) and circulating blood volume by applying hematocrit value to the calculated value of V and Vp.

Results with Discussion

We made a table of these estimations and the values of calculations. As the Table I shows, $\left(\frac{1-K}{Vp}\right)_1$ and $\left(\frac{1-K}{Vp}\right)_2$, and K_1 and K_2 also are almost equal, but a distinct difference is seen in No. 13, and b_1 and b_2 can be considered almost equal too, except in No. 5 and No. 13. Consequently the above theory can be considered right only with a little difference

TABLE

Several Values Calculated, Effective Renal Blood Flow

No.	Sex	Age	Method	$\frac{v(1-K)}{Vp}$	$\frac{1-K}{Vp}$		V (cc.)		R.P.F.
					$\left(\frac{1-K}{Vp}\right)_1$	$\left(\frac{1-K}{Vp}\right)_2$	V_1	V_2	R.P.F.$_1$
1	♂	30	A	3964×10^{-5}	$7{,}44\times10^{-5}$		533		513
2	♂	23	A	3435×10^{-5}	$5{,}31\times10^{-5}$	$5{,}41\times10^{-5}$	647	635	699
3	♂	25	A	3502×10^{-5}	$6{,}00\times10^{-5}$		584		564
4	♂	28	B	3585×10^{-5}	$6{,}97\times10^{-5}$	$6{,}25\times10^{-5}$	514	574	517
5	♂	26	C	3428×10^{-5}	$5{,}24\times10^{-5}$	$6{,}87\times10^{-5}$	654	499	655
6	♂	27	C	3370×10^{-5}	$5{,}88\times10^{-5}$	$7{,}30\times10^{-5}$	573	462	578
7	♂	23	C	4731×10^{-5}	$9{,}64\times10^{-5}$	$8{,}34\times10^{-5}$	491	567	489
8	♂	23	C	4507×10^{-5}	$11{,}36\times10^{-5}$		397		402
9	♂	25	C	4475×10^{-5}	$10{,}31\times10^{-5}$	$12{,}20\times10^{-5}$	434	367	413
10	♂	25	C	4651×10^{-5}	$10{,}37\times10^{-5}$	$12{,}09\times10^{-5}$	449	385	432
11	♂	25	C	3971×10^{-5}	$9{,}91\times10^{-5}$	$11{,}27\times10^{-5}$	401	352	395
12	♂	25	C	3695×10^{-5}	$9{,}23\times10^{-5}$	$10{,}03\times10^{-5}$	398	366	371
13	♂	25	C	3626×10^{-5}	$5{,}51\times10^{-5}$	$8{,}75\times10^{-5}$	676	415	653
14	♂	25	C	3705×10^{-5}	$10{,}79\times10^{-5}$	$10{,}81\times10^{-5}$	343	343	346
15	♂	30	C	4603×10^{-5}	$10{,}66\times10^{-5}$	$9{,}17\times10^{-5}$	432	502	430

caused by experimental deviations. No. 13 shows 653 and 410 cc renal plasma flow according to the method hither to used. This distinct difference shows that there occurred a change in the circulation, and so it is natural that there was a great difference between the two values of K or b. Considering the fast that the effective renal plasma flow shows nearly the same value according to our method and also to the preceding method (v and RPF), we can say that our theory is right.

Comparing the estimation value of the circulating plasma volume of our method (Vp) with that of the method of Gibson *et al.*,[4] the values by these two methods are different in No. 13, as some change can be supposed in circulation in the course of our experiment on this case, but the values are almost the same in all the other cases except in No. 2, 6, 15, where some differences can be seen. Therefore if a little deviation is admitted, our method can estimate circulating blood volume.

Concerning b that is PAH quantity in tissues without any relation to PAH equilibrium between circulating plasma and tissues, b_1 and b_2 are almost equal as we have already stated, and only when this b is assumed, K_1 and K_2 take the same value, so it is certain that there exists such PAH in tissues. As Schwartz[7] says that PAH penetrates into tissue cells, it can be imagined that b shows its value.

I

and Circulating Blood Volume in 15 Healthy Persons

(cc.)	b (mg)		K		Plasma volume		R.B.F. (cc.)		Blood volume (cc.)	$\frac{C_1 Vp}{2000-X_1}$	Ht.
R.P.F.$_2$	b_1	b_2	K_1	K_2	Vp	directly measured	R.B.F.$_1$	R.B.F.$_2$			
	134		0,821		2406	2312	865		3906	0,143	38,4
699	− 83	− 77	0,865	0,862	2542	1969	1097	1076	4308	0,179	41,0
	− 56		0,862		2300	2188	957		3771	0,145	39,0
564	0	− 28	0,847	0,866	2146	2230	886	989	3700	0,155	42,0
495	13	82	0,843	0,794	2996	2812	1038	792	4756	0,144	37,0
466	146	192	0,796	0,747	3469	2953	1005	811	6086	0,129	43,0
562	329	307	0,691	0,733	3205	2953	722	834	4713	0,140	32,0
	179		0,764		2077	2225	522		3709	0,164	44,0
349	47	73	0,827	0,795	1678	1769	761	644	2944	0,161	43,0
370	191	210	0,756	0,716	2353	2100	788	675	4128	0,136	43,0
349	160	179	0,773	0,731	2395	2201	627	550	3738	0,147	36,0
344	352	367	0,696	0,671	3283	3383	711	654	5862	0,157	44,0
410	174	256	0,779	0,650	4011	2876	1325	814	7865	0,127	49,0
333	145	146	0,781	0,782	2020	2044	686	686	4040	0,153	50,0
499	172	150	0,759	0,793	2261	2582	765	884	4002	0,175	43,5

As is already stated, the ratio of PAH volume in plasma and tissues concerning PAH equilibrium, is (1−K): K, and if K is large, the PAH in tissues is large in proportion to plasma PAH. According to Schwartz,[7] inulin space shows the volume of extra-cellular fluid, and PAH space is larger than it, so PAH can be supposed to be distributed into the whole extracellular fluid. And if averaged PAH concentration c_1 in tissues is proportionate to PAH concentration c in circulating plasma and $\frac{c_1}{c}=\alpha$ (constant), we get $\frac{c_1 Vf}{c\,Vp}=\frac{K}{1-K}$, where Vf is the volume of extracellular fluid and Vp is circulating plasma volume, hence,

$$Vf=\frac{K}{\alpha\,(1-K)}Vp$$

Therefore the volume of extracellular fluid can be calculated by finding out α, and if $\alpha=1$, Vf seems to accord with the value of the volume of extracellular fluid in normal condition, so it seems to be certain that α is a value near 1, and we want to decide it in our future study.

This method can be applied directly to tissues in normal or almost normal condition, but we ought to continue our study about how to apply this method when there is serious edema.

Conclusion

Here we have stated our method of simultaneous estimation of effective renal blood flow and circulating blood volume in the normal human body by PAH, and also demonstrated that PAH quantity which penetrates into tissue cells can be calculated in the course of the estimation, and moreover as PAH concentration in plasma and extracellular fluid is perhaps equal in PAH equilibrium, we have discussed at the same time the probability of calculating the volume of extracellular fluid by ascertaining it in our future study.

References

1) Smith, Finkelstein, Aliminosa, Crawford & Graber, J. Clin. Invest., 1945, **24**, 388.
2) Chasis, Redish, Goldring, Ranges & Smith, J. Clin. Invest., 1945, **24**, 583.
3) Warren, Brannon & Merrill, Science, 1944, **100**, 108.
4) Gibson & Evans, J. Clin. Invest., 1937, **16**, 301.
5) Smith, Goldring & Chasis, J. Clin. Invest., 1938, **17**, 263.
6) Alving & Miller, Arch. Int. Med., 1940, **66**, 306.
7) Schwartz, Am. J. Physiol., 1950, **160**, 526.

The Tohoku Journal of Experimental Medicine, Vol. 56, No. 3, 1952

Inunction and Label Methods of BCG Vaccination, an Observation on Schoolchildren

By

Yasaburo Oike and **Jun-ichi Abo**
(大池彌三郎)　(安保順一)

(From the Research Institute for Tuberculosis and Leprosy, Tohoku University, Sendai; and the Medical Clinic, Faculty of Medicine, Hirosaki University, Hirosaki)

Mamoru Sato, Masayo Suzuki and **Seitoku Mizuno**
(佐藤守)　(鈴木正代)　(水野成德)

(From the Research Institute for Tuberculosis and Leprosy, Tohoku University, Sendai)

Ryogo Oyama and **Tatsuo Kasai**
(小山良悟)　(葛西龍郎)

(From the Health Centers in Miyagi Prefecture)

(Received for publication, March 24, 1952)

In BCG vaccination the diminution of local complications and the safety and simplicity of the vaccination technique come into question, in addition to the immunization against tuberculosis. Therefore many vaccination methods have been devised.[1)-9)]

We have contrived what we call the inunction and label methods of BCG vaccination, in which we use neither lancet nor any kind of needle. The inunction method is to rub the mixture of BCG and lanoline into the skin. The label method is to apply an adhesive plaster with the above mentioned mixture to the skin. We have sometimes used the BCG-lanoline together with pilocarpine, which makes the blood-vessels dilate.

Experimental

Preparation of the BCG Vaccines

1) Lanoline-BCG vaccine

The suspension of wet or freeze-dried BCG was mixed with three times as much anhydrous lanoline so that we got lanoline-BCG containing 3.2 to 120 mg. BCG per g.

2) Pilocarpine-lanoline-BCG vaccine

Pilocarpine was added to the above lanoline-BCG vaccine in the

amount of one per cent.

3) Liquid paraffin-BCG vaccine

This vaccine contained 50 mg wet BCG in 1 g. liquid paraffin and was used for inunction.

4) Thick BCG suspension in physiological saline solution

This 50 mg. wet BCG vaccine was used for inunction or scarification.

5) Thin BCG suspension for intractutaneous vaccination

This 0.4 mg. wet or freeze-dried BCG suspension was used as the control.

Among these BCG vaccines, 31 mg. wet BCG-lanoline or BCG-lanoline-pilocarpine vaccine was prepared from 80 mg. wet BCG suspension which had been stored in a refrigerator for 4 months, and so it was a little old.

Vaccination

We vaccinated about 900 tuberculin-negative or -doubtful children, in 5 primary schools, with these BCG vaccines.

1) Inunction method

Every child smeared 0.02 to 0.03 g. of one of the vaccines on its own skin in the border area between the left upper arm and the left armpit or in the left cubital fossa region and rubbed it with the right forefinger or with the curved surface of a bamboo stick, about 100 times, until the skin spot became congestive. Bathing was prohibited on that day and the next one.

2) Label method

We put 0.02 to 0.03 g. of the lanoline or pilocarpine-lanoline vaccine on an adhesive plaster having a diameter of approximately 1 cm. On the skin in the flexor side of the left upper arm we labeled the adhesive plaster, on which we let fall a few drops of collodion to make the plaster stick to the skin. But in not a few cases the plaster came off during the vaccination day or by next morning. Bathing was prohibited on that day and the next one.

We were afraid that the BCG in the ointment vaccines would be controlled by the drops of collodion; which contains alcohol and ether in an abundant volume. In order to solve this problem we carried out an expeiment as follows: Pieces of adhesive plaster with the lanoline-BCG were applied to a glass dish and then were covered with collodion and left standing in a dark place. The pieces, one by one, were cultivated on egg-media over a period of time. We could breed as many colonies as in the control even 25 days after, although BCG colonies decreased a little 38 days after. Fairly many colonies developed even 45 days after. Thus it may be concluded that we can neglect the influence of collodion

on the BCG in our label method.

3) Scarification method as the control

Four crosses measuring about 7 mm. in length were scarified by means of lancet on the outside of left upper arm.

4) Intracutaneous method as the control

On the flexor side of the left upper arm 0.1 cc. vaccine was injected intracutaneously at one point.

Local Complications and Regional Lymph Nodes Affection

We inspected the vaccination spots and regional lymph nodes 43 days to one year and three months after the vaccination, twice at each school.

The intracutaneous vaccination caused a scar in almost every case, and sometimes abscess, ulcer, crust or induration.

The scarification brought about a slight cross scar and sometimes induration.

The inunction and label methods provoked no local change, with the exception of faint pigmentation observed infrequently in the label series. In these series there was no bleeding as noted directly after the intracutaneous injection or scarification.

No regional lymph nodes were palpable in any series.

Tuberculin Reaction

We injected 0.1 cc. tuberculin diluted 1:1000 or 1:2000 intracutaneously on the flexor side of the left upper arm or forearm and measured the redness of the reaction about 24 or 48 hours after the injection, getting an arithmetic average as follows:

Below 4 mm. .....Negative.
5—9 mm. ..... Doubtful.
Above 10 mm. .....Positive.

1) Primary vaccination

Table I shows us that the inunction of 60 or 120 mg. wet BCG-lanoline vaccine provoked the positive conversion of tuberculin reaction at the rate of 22% (14/65) forty-three days after the vaccination. The intracutaneous injection as the control showed 87% positiveness (13/15) forty-three days after. One year and three months after the vaccination the tuberculin reaction turned into positive in 8% (6/72) in the inunction group and in 62% (10/16) in the intracutaneous one.

As shown in Table II, the redness of the reaction was of relatively large size in some cases, and was remarkably clear in many cases.

The other four experiments were done in a similar way. We could

TABLE I

Primary Vaccination (1948–10–26)

Inunction spot: Border area between left upper arm and left armpit.
Inunction with own right forefinger.
Tuberculin reaction: Left upper arm, 24 hours.

Vaccination method	School year	Tuberculin reaction after	
		43 days (dilution, 1:1000)	1 year & 3 months (dilution, 1:2000)
120 mg. wet lanoline-BCG, inunction	2nd	2/23	2/28
	4th	3/10 ……5/33	1/10 ……3/38
60 mg. wet lanoline-BCG, inunction	3rd	1/11	3/15
	5th	8/21 ……9/32	0/19 ……3/34
0.4 mg. wet BCG suspension, intracutaneous	1st	9/10	10/16
	6th	4/5 ……13/15	0/0 ……10/16

TABLE II

Detailed Illustration of the Tuberculin Reaction after 43 Days, given in Table I

Vaccination method	School year	0–4 mm.	5–9mm.	10–14 mm.	15–19 mm.	29–29 mm.	Above 30 mm.	Conversion rate
120 mg. wet lanoline-BCG, inunction	2nd	3	18	1	0	1	0	2/23
	4th	4	3	1	2	0	0	3/10
	Total	7	21	2	2	1	0	5/33
60 mg. wet lanoline-BCG, inunction	3rd	5	5	0	1	0	0	1/11
	5th	4	9	2	4	2	0	8/21
	Total	9	14	2	5	2	0	9/32

not find any difference in the tuberculin conversion between the lanoline-BCG and pilocarpine-lanoline-BCG series, or between the inunction and label methods this time. And the volume of the BCG used, 3.2 to 120 mg. seemed to have nothing to do with becoming tuberculin-positive, probably because of the small number of the vaccinated children in each series. Thereupon we should like to summarize these five sets of data as follows:

In cases of primary BCG vaccination, 20% (73/358) reacted positively to the tuberculin test 43 to 57 days after the vaccination, and 8% (29/350) one year and one month to one year and three months after.

In cases of revaccination, the foregoing vaccination having been intracutaneous, 53% (154/292) became tuberculin-positive 45 to 57 days after, and 13% (34/264) one year and one month to one year and three months after.

The inunction and label methods could produce tuberculin positive more in the revaccination than in the primary vaccination.

The tuberculin conversion in the inunction and label series was less compared with that in the control series, namely, in the intracutaneous and scarification series. We suppose we shall be able to increase the rate of the tuberculin conversion in the inunction and label methods, by using thicker BCG ointment and by spreading the ointment on the skin over a wider area.

There were many children who had reacted negatively to the tuberculin test before the inunction or label and who became tuberculin-doubtful after the inunction or label. This fact will show us that the inunction or label of the BCG ointment somehow caused tuberculin allergy, though weak, in these children. There were few children who had reacted doubtfully before the inunction or label and who became tuberculin-negative after the inunction or label.

The greater part of our study was done on six- to eight-year-old schoolchildren, and only a small part on nine- to eleven-year-olds. It was confirmed that the older the children, the more of them became tuberculin-positive. An investigator[10] has noted this. But in our cases this was partly due to the poor inunction process by the younger children.

We applied the 50 mg. wet BCG-liquid paraffin or 50 mg. wet BCG suspension in physiological saline solution to schoolchildren by inunction, resulting in the tuberculin conversion in 1/15 or 1/13 forty-eight days after and in 1/15 or 0/12 one year and one month after.

Of 72 children who had been given the inunction of lanoline-BCG previously and who nevertheless had been tuberculin-negative, 27 became tuberculin-positive 45 to 57 days after the revaccination by means of the inunction of lanolin-BCG, and 2 of 61 were positive one year and one month after.

Of 12 who had been vaccinated with lanoline-BCG by means of inunction and had been tuberculin-negative, 5 became tuberculin-positive 57 days after the revaccination by means of the inunction of pilocarpine-lanoline-BCG, and none of 10 was positive one year and two months after.

Discussion

We could vaccinate children with BCG by means of inunction or label of the mixture of BCG and lanoline or of the mixture of BCG, lanoline and pilocarpine. Now there arises the question whether the BCG passed through the local skin applied with lanoline-BCG or pilocarpine-lanoline-BCG or not, because the ointment on the skin was not cleaned up after the inunction or label and the adhesive plaster with BCG was not specially attended, and so the BCG on the skin or the adhesive plaster would be spread around and could enter through the nose, mouth and/or elsewhere

to provoke the tuberculin allergy.

In order to set the question at rest, we tested tuberculin on both upper arms. The tuberculin reaction on the vaccinated left upper arm was larger in size than that on the non-vaccinated right upper arm. From this fact we can conclude that the BCG also entered through the applied skin portion, because the nearer the site of tuberculin injection is to the site vaccinated with BCG, the larger is the size of tuberculin reaction.

And, since the tuberculin reaction examined in these cases was not so large in size and the tuberculin conversion was relatively frequent, it is clear that the tuberculin conversion was brought about by the BCG, not by spontaneous tuberculous infection.

It is well known that bacteria or various things pass through human skin into the body. The skin is not always a barricade between the outside and inside.

Oxygen is taken into the body through human skin in skin respiration.

Toilet powder containing lead brings on lead posisoning because the lead enters through the skin.

When we apply mercurial ointment by inunction in the treatment of syphilis, the mercurial particles pass through the skin.

Furuncle is set up by invasion by some cocci etc. into glandula sebacea or glandula sudorifera.

Spirochaeta pallida, which is self-moving, unlike the above mentioned cocci, passes through microscopic skin lesions.

The larvae of ankylostoma and schistosoma are capable of penetrating skin.

Gall-bladder is essentially competent for apparent absorption of water and is not skin but mucosa, and so it may be unreasonable to cite here the fact that gall-bladder contains many ascaris eggs in its wall. Though they are relatively large, they are taken into the wall.[11] This is analogous to the passage of various materials through the skin.

Verruca necrogenica is caused by the entrance of tubercle bacilli through the skin. On this occasion there must be some fine lesion on the local skin, it is said. On the other hand Kato[12][13] and Niwase[14] observed the passage of tubercle bacilli through uninjured healthy skin of guinea pigs and tuberculous lesions at autopsy. Kato and Koenigsfeld[15] demonstrated that tubercle bacilli could also enter through injured skin.

We applied lanoline-BCG or pilocarpine-lanoline-BCG to rabbits by inunction or label and could stain the BCG in the skin.[16]

Our inunction method is analogous to scarification or multiple puncture methods which injure the skin with lancet or needle because we rub the skin to make microscopic lesions on the skin. Our label method is

similar to the above mentioned methods of Kato or Niwase of scattering BCG vaccine with a glass stick or smearing it with a hair-pencil because no lesion arises mechanically either in our method or Kato's or Niwase's. But we must keep in mind that the labeled adhesive plaster with lanoline-BCG may provoke some change of the skin with the lapse of time.

Conclusion

It is possible to vaccinate schoolchildren with BCG by means of inunction or labeling of lanoline-BCG or pilocarpine-lanoline-BCG vaccines without any local complications, although the rate of tuberculin conversion is not so satisfactory.

References

1) Calmette, Bull. Acad. Méd. Paris, 1931, **106**, 37; Zbt. Tbc., 1932, **65**, 10.
2) Weill-Hallé and Turpin, Bull. Acad. Méd. Paris, 1929, **97**, 126.
3) Wallgren, J. Am. Med. Assoc., 1928, **91**, 1876; Acta Tbc. Scan., 1933, **7**, 72.
4) Rosenthal, Am. Rev. Tbc., 1937, **35**, 679.
5) Chaussinand, La vaccination contre la tuberculose par le BCG, Paris, 1931, p. 147.
6) Bacanu, C. r. Soc. Biol., 1938, **129**, 1017.
7) Weill-Hallé, Bull. et Mém. Med. Hôp. Paris, 1938, **54**, 1647.
8) Toda and Shimamoto, The 13th Rengo-Biseibutsu-Gakkai-Kiroku, 1939.
9) Yoshida, Kekkaku, 1945, **18**, 1261.
10) Shigemura, Nippon Igaku, 1944, No. 3390, 879.
11) Maki, Tokyo Med. J., 1952, **69**, 6.
12) Kato, Kekkaku, 1927, **5**, 492.
13) Kato, ibid., 1928, **6**, 65.
14) Niwase, Osaka-i-shi., 1932, **31**, 3303.
15) Koenigsfeld, Zbt. Bak., 1911, **60**, 28.
16) Oike *et al.*, Rep. Res. Inst. Tbc. and Lep., Tohoku Univ., in press.

The Tohoku Journal of Experimental Medicine, Vol. 56, No. 3, 1952

Chromaticity Diagrams and Retinal Color Processes

By

Yasuhiro Arakawa and **Toshihiko Oikawa**
(荒川 安廣) (及川 俊彦)

(From the Physiological Laboratory of Prof. K. Motokawa,
Tohoku University, Sendai)

(Received for publication, April 7, 1952)

Introduction

Newton devised a circular color table to give a graphical representation to his conception on the color mixture. Subsequently Maxwell noticed that in the color mixture, the spectral colors could not be arranged on a circle, but that they had to be placed on the straight sides of a triangle.

Thus Maxwell, Helmholtz and others determined a spectral color triangle on the basis of the color mixing system by using the red, green and blue spectral lights as the three primaries.

König and Dieterici,[1] Abney[2] and others determined the so-called primary sensation curves, which were studied in further details by many investigators such as Exner, Ives, Haschek[3] and others. These curves gave rise to a misunderstanding among some physiologists and physicists that they were a certain expression of physiological color processes involved, but in reality they are nothing but a product of a mathematical treatment of the data obtained from color mixing experiments.

It is certain that such a treatment is based, to a certain extent, on the trichromatic theory of Young-Helmholtz and that the trichromatic system is useful as a framework for color specification, but it does not follow from those facts that there exist three spectral physiological processes which would correspond to the scheme of the trichromatic theory.

Recently Motokawa[4][5] derived three curves very similar to the primary sensation curves from the data obtained by means of his newly devised physiological method and constructed a color diagram which closely resembles the usual chromaticity diagram. It deserves attention that a color diagram has been constructed from physiological data without resort to any color mixing procedure.

His diagram is, however, based on the data of only one subject. Therefore, we intended in the present investigation to confirm his result

on other subjects and to clarify the significance of such physiological color diagrams by comparing them with the usual chromaticity diagram based on the color mixture.

Experimental

Method and Results

The method used was essentially the same as that employed by Motokawa.

A pair of silver electrodes of 2×1.5 cm. in size, one on the forehead slightly above the eyebrow, and the other on the homolateral temple of the subject served for electrical stimulation of the eye. The stimulating current used was a single constant current pulse of 100 msec. in duration. An opaque glass disc of 1 cm. in diameter, which was illuminated from behind by monochromatic light from a spectroscope, was viewed from a distance of 30 cm., the visual angle subtended by the disc at the eye being 2°.

The disc was centrally fixated in all our experiments. The energy of monochromatic light was always kept constant by adjusting the width of the collimator slit to prescribed values computed from the spectral energy distribution of the light source.

The object of measurements was the effect of monochromatic illumination which was measurable as increases in electrical excitability of the retina following the illumination. Increases in electrical excitability were measured in the following manner. After preliminary dark adaptation of about 20 minutes an electrical pulse was passed through the eye so as to cause a distinct electrical phosphene. The voltage was then reduced step by step until the subject could no more distinguish the stimulus in question from one far below the threshold. One value of threshold could usually be determined after about 20—40 repeated trials. Thus the threshold at the resting level of the eye was determined. Then, similar measurements were carried out at 1, 1.5, 2 and 3 seconds after termination of a 2 sec. illumination by monochromatic light. The difference of excitabilities, reciprocals of threshold values determined with and without pre-illumination was expressed in percentage of the resting excitability and denoted by ζ.

As was shown by Motokawa,[4] ζ-time curves for red, yellow, green and blue lights had a maximum at about 1, 1.5, 2 and 3 seconds after cessation of the pre-illuminating light. The crest times which characterize the pre-illuminating light depended only on the wave-length, but not on the intensity and duration of the light. The height of the maximum, however, increased in proportion to the logarithm of the intensity within

a wide range of moderate intensities.

According to Motokawa's analysis[5] ζ-values at 1, 1.5, 2 and 3 seconds after illumination indicate the excitation of the red, yellow, green and blue receptors respectively. If this interpretation is correct, the spectral distribution of ζ as determined, say—at 3 seconds after illumination—should give a spectral distribution of the excitability of the blue receptor.

Based on this principle, we determined ζ-values after 2 sec. exposure of the eye to various spectral lights, the interval between the end of illumination and the electrical stimulus being fixed at 3 seconds, and thus the spectral distribution curve B in Fig. 1 was mapped out. Similarly the curves G, Y and R were obtained by fixing the interval at 2, 1.5 and 1 seconds respectively.

For comparison, Motokawa's result is represented as the set of curves (M.) in Fig. 1. The other sets, (A.) and (O.) were obtained in the present experiments from subjects A. and O. respectively.

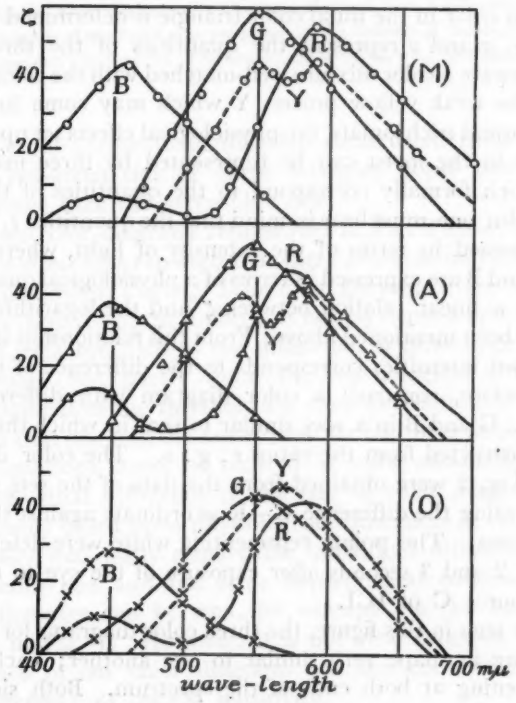


Fig. 1. Physiological sensation curves or response curves from 3 different individuals. Explanation in. text.

As can be seen in this figure, the corresponding curves obtained from the three different subjects closely resemble one another not only in shape but also with respect to the location of the maxima.

However, the curves Y in (M.) and (A.) show two humps at the wavelengths corresponding to the maxima of the curves G and R, whereas the Y curve in (O.) has only one maximum at the yellow part of the spectrum. Motokawa[5] interpreted the Y curve in (M.) to be composed of the two processes R and G, but afterwards it was revealed by further work with an illuminating patch of much reduced visual angle that there exists an independent weak yellow process at the fovea of the human eye.[6][7] Therefore the curve Y in (O.) may represent the distribution curve for an independent yellow process.

Physiological color diagram

For specification of colors any color is expressed geometrically by a point on a two-dimensional color diagram such as a color triangle. The coordinate of a color in the usual color triangle is determined by the ratios $r:g:v$, where r, g and v represent the quantities of the three primaries required to prepare a color mixture well-matched with the color in question. Apart from the weak yellow process Y which may come into action at the fovea of normal trichromats, the physiological effects set up by a colored light stimulus in the fovea can be represented by three magnitudes R, G and B, which formally correspond to the quantities of the primaries r, g and v. But one must bear in mind that the quantities r, g and v are generally expressed in terms of the intensity of light, whereas our magnitudes R, G and B are expressed in terms of a physiological quantity. However, there is a linear relation between ζ and the logarithm of light intensity, as has been mentioned above. From this relationship it follows that the ratio of two intensities corresponds to the difference of two ζ-values. We can, therefore, construct a color diagram with differences of our magnitudes R, G and B in a way similar to that in which the usual color triangle is constructed from the ratios $r : g : v$. The color diagrams represented in Fig. 2 were obtained from the data of the sets of curves in Fig. 1, by plotting the difference G—R as ordinate against the difference B—G as abscissa. The points representing white were determined from ζ-values at 1, 2 and 3 seconds after exposure of the eye to the standard white light source C of ICI.

As can be seen in this figure, the three color diagrams for three different subjects are in shape very similar to one another; each of them is a triangle, opening at both ends of the spectrum. Both sides of these triangles are concave towards the point representing white, and the spectral order from blue to red is never retrogressive. These properties are found

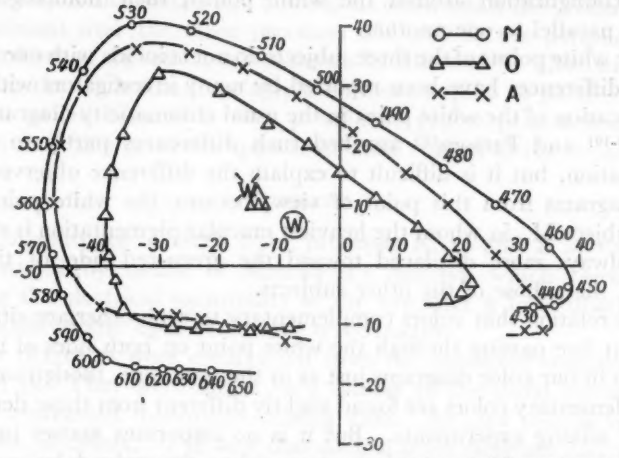


Fig. 2. Physiological color diagrams obtained from 3 different individuals. Wave-lengths of spectral lights are given on the contour of the largest diagram. W's represent white points. Ordinates: difference G—R. Abscissas: difference B—G.

also in the usual chromaticity diagram constructed from color mixture data. The red-green side of these triangles slopes more steeply and is more concave than the green-blue side.

The three color diagrams represented in Fig. 2 show, however, some differences in size. It is reported that there are wide individual differences in normal color vision (Pierce,[8] Tcherning, Larson, Gibson and Tyndall[8]). According to their views, the differences largely depend upon the individual character of each eye such as the difference of physiological elements of visual function, but partly upon the psychological elements such as the final judgment of the threshold for color perception. Wright,[9] however, reports that one and the same result for the observers under the most favorable conditions could be secured in color measuring experiments.

The individual difference of the size of color diagrams mentioned above is possibly based upon physiological differences in retinal excitation, for we usually find fairly remarkable individual differences in ζ-values obtained under one and the same experimental conditions. Such individual differences can be seen, for example, in the heights of curves represented in Fig. 1. However, it is not clear whether or not such a size difference of our color diagrams has any connection with the individual difference of optical sensitivity.

It is further to be noted that the three color diagrams are in con-

centric configuration around the white points, their homologous sides running parallel to one another.

The white points of the three subjects do not coincide with one another. Similar differences have been reported by many investigators with regard to the location of the white point in the usual chromaticity diagram. Wright[8)-10)] and Parsons[11)] ascribed such differences partly to macular pigmentation, but it is difficult to explain the difference observed in our color diagrams from this point of view, because the white point of the oldest subject M., in whom the heaviest macular pigmentation is expected, is not always more displaced toward the green-red side of the color triangle, than those of the other subjects.

The relation that colors complementary to each other are situated on a straight line passing through the white point on both sides of it can be seen also in our color diagrams just as in the usual one, though some pairs of complementary colors are found slightly different from those determined in color mixing experiments. But it is no important matter in view of the circumstance that complementary colors determined by various investigators such as Helmholtz,[12)] v. Frey and v. Kries,[13)] König and Dieterici,[1)] Angier and Trendelenburg[14)] and others show no exact agreement with one another.

Discussion

As has been shown above, our physiological color diagram bears a striking resemblance to the usual color diagram obtained colorimetrically. This fact suggests that the three-color system has really a certain physiological basis as the Young-Helmholtz's theory advocated. But this does not always imply that the trichromatic theory is valid in a strict sense. As has been stated above, Motokawa *et al*[6)7)] proved the existence of an independent yellow process at the fovea of normal trichromats, though the development of this process is generally weaker at the fovea than that of the other processes. In construction of our physiological color diagrams, the yellow process was not taken into account, because no two-dimensional diagram can be constructed with 4 components R, Y, G and B. Nevertheless, our color diagrams constructed in this way have proved to represent almost all characteristics of the usual chromaticity diagram and to be as useful as the latter for specification of colors. This situation indicates that the foveal color vision of normal trichromats is essentially trichromatic, and at the same time it may be concluded from our finding that the yellow process plays only an accessory role at the fovea of normal trichromats. Thus, the trichromatic theory has an approximate validity so far as the normal foveal vision at moderate light intensities and within a certain range of visual angle is concerned.

However, in dichromats and anomalous trichromats the yellow process is predominant over the other processes not only in the periphery but also at the fovea of the retina, as was shown by Ebe *et al.*[15] Even in normal trichromats the trichromatic principle no more applies to parafoveal and peripheral vision, because a very prominent yellow process besides the red, green and blue processes appears in these regions of the retina.

Summary

The magnitudes of three color processes R, G and B at the fovea were measured by means of Motokawa's method, which consisted of measuring the electrical excitability of the eye after a brief illumination with monochromatic light of various wave-lengths.

Color diagrams were constructed from the measured magnitudes of R, G and B. They were found very similar to the usual chromaticity diagram constructed from color mixing data. So they can be used for specification of colors. The fact that such a color diagram can be constructed from three physiological quantities suggests that the normal foveal vision is essentially trichromatic. The limitation of the validity of the trichromatic principle was further discussed on the basis of physiological data.

We wish to thank Prof. K. Motokawa for his good advice and cordial guidance.

References

1) König, A. and Dieterici, C., Ztschr. f. Psychol. u. Physiol. d. Sinnesorg., 1893, **4**, 241.
2) Abney, W., Researches in colour vision and the trichromatic theory, London, 1913, p. 223.
3) Halbertsma, K.T.A., A history of the theory of colour, Amsterdam, 1949, p. 200.
4) Motokawa, K., J. Neurophysiol., 1949, **12**, 291.
5) Motokawa, K., ibid., 1949, **12**, 465.
6) Motokawa, K., Ebe, M., Arakawa, Y. and Oikawa, T., Nature, 1951, **167**, 729.
7) Motokawa, K., Ebe, M., Arakawa, Y. and Oikawa, T., Jap. J. Physiol., 1951, **2**, 50.
8) Pierce, W. O'D., Gt. Brit. Med. Res. Council, Sp. Rep. Ser., 1933, No. 181.
9) Wright, W. D., Researches on normal and defective colour vision, London, 1946, p. 153.
10) Wright, W. D., Gt. Brit. Med. Res. Council, Sp. Rep. Ser., 1929, No. 139.
11) Parsons, J. H., An introduction to the study of colour vision, Cambridge, 1924, p. 41.
12) Helmholtz, H., Handb. Physiol. Optik, Hamburg and Leipzig, 1896, p. 316.
13) v. Frey, M. and v. Kries, J., Arch. f. Anat. u. Physiol., 1881, Physiol. Abthlg. 336.
14) Angier, P. and Trendelenburg, W., Ztschr. f. Psychol. u. Physiol. d. Sinnesorg., 1905, **39**, 284.
15) Ebe, M., Isobe, K. and Motokawa, K., Science, 1951, **113**, 353.

The Tohoku Journal of Experimental Medicine, Vol. 56, No. 3, 1952

Chemistry and Biology of Lipids

XI. Group A Lipoid of Human Pancreas

First Paper

By

Masahiro Tokura

(登倉 正博)

(From the Medico-chemical Institute, Tohoku University, Sendai; Director: Prof. H. Masamune)

(Received for publication, April 8, 1952)

A Group lipoid was separated from pancreases of persons belonging to Group **A** in an electrophoretically homogeneous state. The pancreases dehydrated with cold alcohol were treated with hot alcohol and the extract at first fractioned by various organic solvents, which, however gave merely a mixture of several components as an active fraction. Hence, this was next subdivided by means of lead acetate and the product which was still heterogeneous was subjected to counter current distribution. A substance to the end in view was thus obtained. It inhibited the isoagglutination of **A** erythrocytes at a dilution of 1:100,000. Biuret-negative, Elson & Morgan (tested on an acid hydrolysate)- and Molisch-positive. It was somewhat soluble in benzene and water. Analyses: N 2.2%, P 2.6%, ferricyanide reduction after hydrolysis 17.7% as glucose, ash 9.4%. Details will be given in the experimental part.

Experimental

Preparation and Some Properties of the Product

Fractionation was carried out as follows, taking the anti-isohemoagglutinative potency and electrophoretic pattern of the divisions as indications. The method of the serological assay was the one usual to us.

Fresh pancreases from 20 Group **A** individuals (1626 g.) were masticated, dehydrated by placing in 4 volumes of denatured alcohol containing 3.5% methanol for longer than 1 week and further dried by means of a Faust apparatus.

Stage 1. The material obtained (179 g.) was powdered and refluxed with 900 cc. of the denatured alcohol for 2 hours. The solution was filtered while hot and the residue again treated with the same volume of the denatured alcohol. (The residue here amounted to 155 g. when

dried up.) The solutions were united and placed in an ice-chest for 2 days, and the sticky yellow precipitate, that had occurred in the mean time, was separated and washed with acetone. A small quantity (0.05 g.) of a coarse yellowish powder was given. This was found not sero-reactive (not anti-isoagglutinative to **A** erythrocytes) at a dilution more than 1:4,000. The clear yellow brown mother fluid was distilled in vacuo and the syrupy remainder also washed with acetone thoroughly. A yellowish hygroscopic powder given here (3.7 g.) was Group **A** active at a dilution of 1:80,000 (On distillation of the acetone washings were left 3.9 g. of a brown oily fluid). It was next fractioned employing four 40 cc. portions of dry ether. The insoluble part—a hygroscopic, yellowish, fine powder—weighed 3.5 g. and was group-active at a dilution of 1:160,-000. The ether-soluble recovered by distillation under diminished pressure had yellow butterlike appearance and showed no prevention of the agglutination at 1:800 and caused hemolysis at dilutions of 1:400 and less. Yield, 0.2 g. It was next tried to subdivide the ether-insoluble with cold benzene. The benzene-soluble (a trace) recovered as above was group-active at a dilution of only 1:1,000. The main insoluble part (a yellowish brown powder; 3.5 g.; the sero-reactive dilution 1:400,000) was found again hardly soluble in petrol ether (cold) and the potency was not elevated thereby. Finally it was kneaded with changes of cold chloroform. Of 3.5 g. of the substance about 1/10 dissolved. The still residue of the solutions (a brown coarse powder, 0.33 g.) was perfectly inhibitory to the isoagglutination of **A** erythrocytes at 1:1,000 dilution but only partially when the dilution was increased tenfold. The insoluble part (a yellowish brown, slightly hygroscopic, coarse powder; 3.1 g.) exhibited the anti-isohemoagglutinative power same as before the division. This (Prep. I) gave the positive biuret, ninhydrin, Sakaguchi (for arginine), indirect-Osaki & Turumi and Molisch reaction. The direct-Osaki & Turumi reaction was not shown. The solubilities and analyses are embodied in Tables I and II.

Electrophoretic analysis demonstrated the extreme inhomogeneity of the substance (Fig. 1).

Remarks on Prep. I. Taking small portions of the substance, attempts were made to see if further processing of resembling operations serves the purpose.

1) Division by the aid of hot benzene gave fractions similar to each other in the anti-isohemoagglutinative potency.

2) A portion of the substance was refluxed with changes of ethanol (Most of it went into solution) and the solutions were cooled in an ice chamber. The occurring precipitates were separated by centrifugation and the supernatant fluids condensed together to 1/5 the total volume and chilled as before, followed by separation of the deposit. The part in the mother fluid here was recovered by distillation. The deposit from the condensate was found most sero-reactive (active at a dilution of 1:800,000) but its amount reached less than 1/10 of the

TABLE I

Solubilities of Prep. I

−: insoluble, ±: faintly sol., +: partly sol., ++: mostly sol., ###: completely sol.

Solvent	Water		Methanol		Ethanol		Glacial acetic acid		Ether		Petrol ether		Benzene		Chloroform		Tetralin		Pyridine	
	cold	hot	cold	hot	cold	hot	cold	hot	cold	hot	cold	hot	cold	hot	cold	hot	cold	hot	cold	hot
Solubility	++*### †		###	###	+	++	+	+	−	−	−	−	±	+	−	−	+	###	±	±

* An opalescent solution was given. Variation of the pH to 8.0 and to 1.2 did not change the solubility. † A clear solution formed.

TABLE II

Composition of Prep. I

Analysis	Per cent
N*	9.1
P†	2.3
Reduction on ferricyanide of the hydrolysate as glucose‡	11.5
Ash§	6.6

* Micro Kjeldahl. † A modification of Plimmer method[1]. ‡ Masamune and Tanabe method[2]. The hydrolysate (filtrate) contained none precipitable by phosphotungstic acid so that it was immediately neutralized to measure the ferricyanide reduction. § Pregl method without the use of H_2SO_4.

Fig. 1

Fig. 2

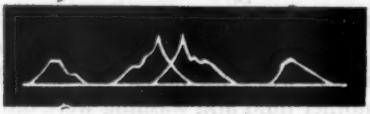


Fig. 1. An electrophoretic pattern of a 1% solution (opalescent) of Prep. I in phosphate buffer, pH 7.8, I 0.29; current 20 ma; temperature 17°C. Exposure 5/6 hour after starting current. Ascending limb to the left.

Fig. 2. An electrophoresis pattern of a 1% solution (opalescent) of Prep. I′ in a phosphate buffer as above; current 19–20 ma; temperature 15°C. Exposure 40 minutes after starting current. Ascending limb to the left.

material started with. The last and least-potent fraction, that occupied 7/10 part of the material, completely inhibited the isohemoagglutination until it was diluted up to 1:100,000.

3) 200 mg. of the substance were washed with cold ethanol, ether and benzene in succession and finally taken up in cold methanol and the centrifuged clear supernatant was

distilled to dryness, followed by washing with acetone. 35 mg. of a yellowish fine powder were given—Prep. I′. This was specifically inhibitory to the isoagglutination of **A** erythrocytes at a dilution of 1:800,000. Molisch positive, biuret negative. Thus the serological potency was elevated and the biuret-giving contaminant was eliminated, but the electrophoresis revealed the heterogeneity of it (Fig. 2). It formed an opalescent solution in water, and addition of acetic acid (to pH 2.0) rather made the solution less turbid.

On the other hand, the alcoholic washings above were distilled in vacuo to dryness and washed with acetone. A yellow powder left (137 mg.) was group-active at a dilution of 1:200,000 and gave the positive biuret reaction in addition to the positive Molisch. 70 mg. of it were further treated with cold butanol and the insoluble was washed with acetone. 32 mg. of a yellow fine powder were obtained, which possessed the same potency as before the division, and Molisch and biuret test resulted in positive. The still residue of the butanol-solution was also washed with acetone—Prep. I″ (23 mg., a yellow fine powder). This was as active as the insoluble. Molisch positive. Although it did not give the biuret reaction, the electrophoresis suggested three components (Fig. 3).

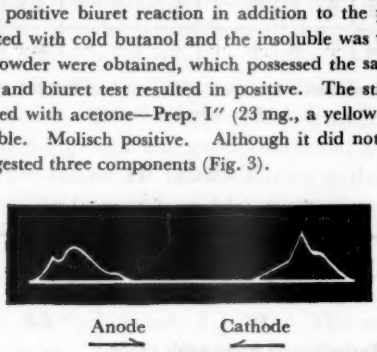


Fig. 3. An electrophoretic pattern of a 0.8% solution (a little opalescent) of Prep. I″ in phosphate buffer, pH 7.8, I 0.29; temperature 19°C; current 20 ma. Exposure 40 minutes after starting current. Ascending limb to the left.

Since these operations proved far from sufficient, metal salts were examined, of which lead acetate appeared pretty expedient.

2nd stage. 600 mg. of Prep. I were taken up in 60 cc. of water and 6 cc. of a 5% solution of lead acetate (crystalline) were added (pH of the mixture was 4.8), whereby precipitation occurred. Centrifuged.

a) The supernatant was bubbled with H_2S gas and the precipitate hereby was centrifuged and discarded after washing with hot water. The mother fluid and washing were united, neutralized to pH 5.0 with NaOH, distilled under reduced pressure to dryness and washed with acetone. 350 mg. of a yellowish, hygroscopic, fine powder were given (Molisch and biuret positive). It was devoid of the anti-agglutinative activity (tested at a dilution of 1:100).

b) The lead acetate-precipitate was washed with a very dilute solution of the salt and then 70 cc. of water added. The suspension was acidified to pH 2.0 with HCl and H_2S passed. And the centrifugate (dark brown) was washed with a very dilute HCl of pH 2.0. i) The centrifuged supernatant was neutralized to pH 6.0, distilled in vacuo to dryness and washed with alcohol. 73 mg. of a yellowish fine powder were obtained, which did not inhibit the isoagglutination of **A** erythrocytes

(examined at a dilution of 1:100) and gave neither Molisch nor biuret reaction. The part, that had dissolved in alcohol on washing, was recovered by distillation and washed with acetone. The substance here (220 mg.) was again found sero-inactive (at a dilution of 1:100). ii) The H_2S-precipitate was extracted with changes of methanol until the residue became non-sticky. The solutions combined (pH 5.6) were distilled in vacuo to dryness and again treated with methanol, followed by evaporation of the solutions and washing with acetone. 80 mg. of a white non-sticky, non-hygroscopic powder were yielded. This was specifically inhibitory to the isoagglutination of **A** erythrocytes at a dilution of 1:1,000,000—Prep. II. Molisch positive, biuret negative. Phosphorus was detected. As is illustrated in Table III, it was scarcely soluble in plain and faintly-alkalinized water, but completely soluble in glycol, methanol and ethanol. Therefore, for its electrophoresis was at first a mixture of glycol and phosphate buffer of pH 7.8 (1:1 by volume) em-

TABLE III

Solubilities of Prep. II

−: insoluble, ±: faintly sol., +: partly sol., ++: mostly sol., +++: completely sol.

Solvent	Water		Glycol	Glycerol	Methanol	Ethanol	Ether	Petrol ether	Benzene	Chloroform	Tetralin
	plain	alkaline*									
Solubility	±	±	+++	±	+++	+++	−	−	±	++	−

* A suspension of the substance in water was alkalized to pH 9.6.

ployed as a solvent, but the boundary moved too slowly. Although several other solvent mixtures were also tested, all proved useless so that the writer was compelled to apply a dilute solution in that phosphate buffer to this analysis.

Electrophoretic analysis. See Fig. 4.

320 mg. of a similar product was prepared, besides, from 2.1 g. of Prep. I.

Fig. 4. An electrophoretic pattern of a 1/3% solution (turbid) of Prep. II in phosphate buffer, pH 7.8, I 0.29; temperature 19°C; current 20.5 ma. Exposure 25 minutes after starting current. Ascending limb to the left.

Remark. Prep. I was precipitated from water neither by $BaCl_2$ nor by $CaCl_2$.

3rd stage (*counter current distribution*). 9 transfer distribution was made, using separatory funnels. The solvent pair consisted of an equilibrated mixture of water, methanol, benzene and toluene (1:4:4:1 by volume). 200 mg. of Prep. II were dissolved in 100 cc. of the lower phase, shaken with an equal volume of the upper phase and the centrifuged upper layer was transferred to a second funnel (By standing alone a long time was required for clear-cut separation of the layers). After the last transfer the contents of each of the funnels were also shaken thoroughly. When the distribution was completed, a 0.5 cc. portion of all the phases was evaporated by placing in a vacuum desiccator and 0.5 cc. of physiological saline added for the assay of the group activity (anti-isoagglutinative force to **A** erythrocytes):

	Number of funnels									
	0	1	2	3	4	5	6	7	8	9
Upper phase	##	##	+	+	+	+	+	−	−	−
Lower phase	##	##	##	+	+	+	+	+	+	−

Control (without any of the substances) ##

−: no agglutination, +: slight aggl., ##: marked aggl., ###: strong aggl.

The **B** serum had agglutinin titre 320 and was diluted twenty times.

Then the active solutions were progressively diluted for more accurate measurement:

		Dilution of the specimens						
		1:1	1:10	$1:10^2$	$1:10^3$	$1:10^4$	$1:5\times10^4$	$1:10^5$
Upper phase: Funnel	7	−	−	+	+	+	+	+
	8	−	−	−	+	+	+	+
	9	−	−	−	−	+	+	+
Lower phase: Funnel	9	−	+	+	+	+	+	+

Control (without any of the substances) ##

The upper phase of Funnels 7, 8 and 9 were combined and poured in small portions into a vacuum distillation flask, and the still residue washed with acetone and dried. The substance given here (55 mg.) was taken up in water by slight warming (40–50°C, 5 minutes) and the centrifuged supernatant distilled in vacuo to dryness and washed with acetone. The product (a white non-sticky fine powder)—Prep. III—amounted to 50 mg. It specifically inhibited the isoagglutination of **A** erythrocytes at a dilution of 1:100,000, that is, it was much less active than before the distribution process. The cause of this decrease is uncertain at present. The activity was not returned by mixing the product with the almost inactive water-insoluble part. Elson & Morgan (tested on a hydrolysate

by 1 *N* H_2SO_4) and Molisch positive. Neither Goldschmiedt nor Neuberg & Saneyoshi nor Rosenthaler reaction was shown. The solubilities and analyses are recorded in Tables IV and V.

TABLE IV

Solubilities of Prep. III

−: insoluble, ±: faintly sol., +: partly sol., ++: mostly sol.

Solvent	Water		Methanol		Ethanol		Glacial acetic acid		Ether		Petrol ether		Benzene		Chloroform		Tetralin	
	cold	hot	cold	hot	cold	hot	cold	hot	cold	hot	cold	hot	cold	hot	cold	hot	cold	hot
Solubility	+*	++	±	+	±	±	−	±	−	−	−	−	+†	+	±†	±	+†	++

* The solution was opalescent. † The undissolved particles swelled up and took a jellylike state.

TABLE V

Composition of Prep. III

Analysis	Per cent
N*	2.2
P†	2.6
Reduction on ferricyanide of the hydrolysate as glucose‡	17.7
Ash§	9.3

*, †, ‡, § Signify the same as in Table II.

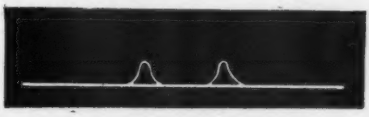


Fig. 5. An electrophoretic pattern of a 4/7% solution (opalescent) of Prep. III in phosphate buffer, pH 7.8, I 0.29; temperature 8°C; current 10 ma. Exposure 52 minutes after starting current. Mobility 9.0 $cm^2 \cdot volt^{-1} \cdot sec^{-1} \cdot 10^5$. Ascending limb to the left.

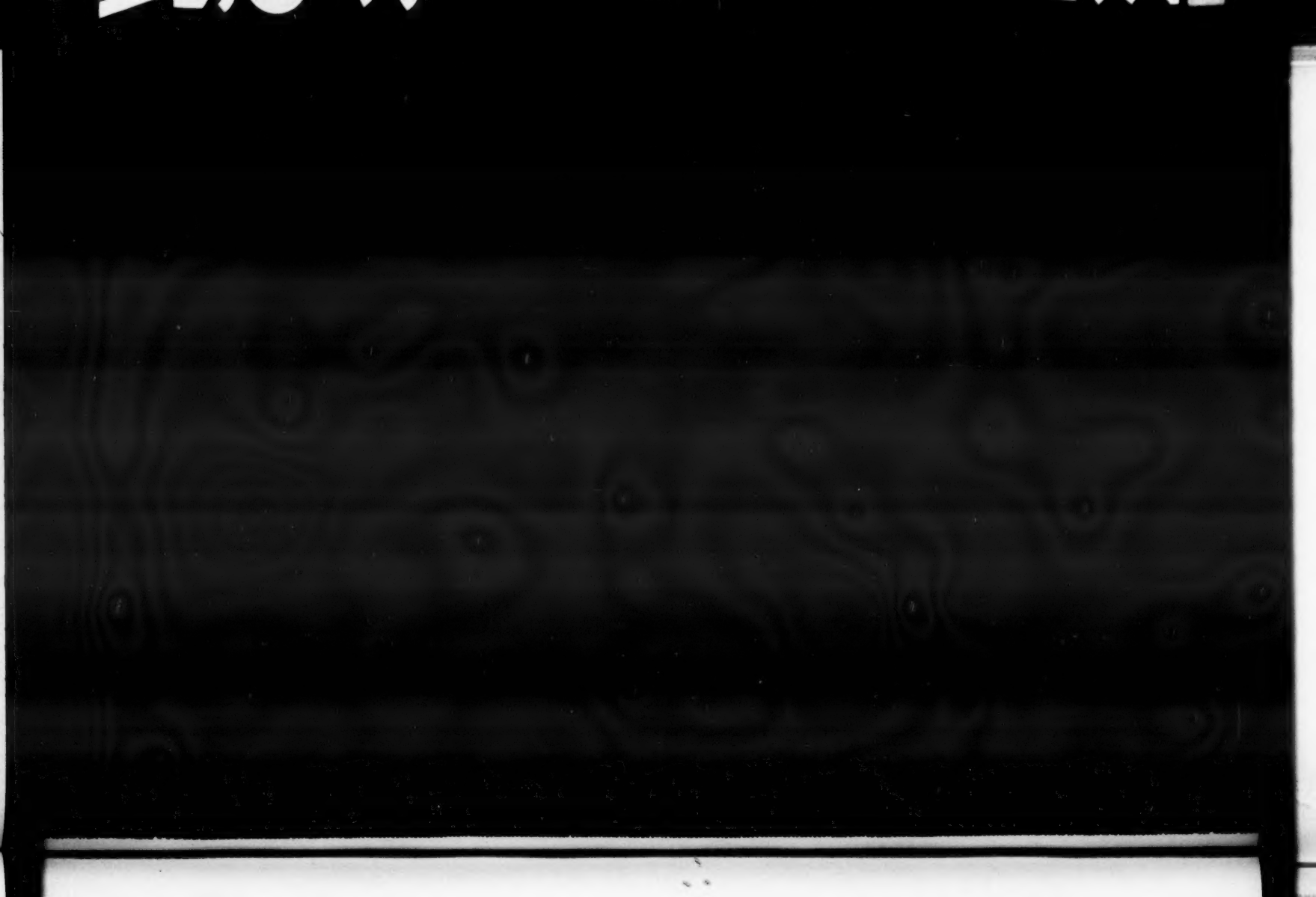
Electrophoresis demonstrated only one component (Fig. 5).

SUMMARY

A method of preparing the Group A lipoid of human pancreas has been devised. A rough investigation was also made regarding the com-

position and physical properties of the product.

The work was aided by a grant, given from the Education Department through the Grant Committee for Scientific Researches. H. Masamune.

References

1) Haneda, Sinokawa and Yasuoka, Tohoku J. Exp. Med., 1944, **46**, 263.
2) Masamune and Tanabe, J. Bioch., 1938, **28**, 19.